EFFECT OF NITRATE REDUCTION ON THE METHANOGENIC FERMENTATION: PROCESS INTERACTIONS AND MODELING

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EFFECT OF NITRATE REDUCTION ON THE METHANOGENIC FERMENTATION: PROCESS INTERACTIONS AND MODELING

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Dedicated to people whom I love the most,

my parents, my sister, and Utku Karnabat

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SUMMARY

Combined treatment technologies for the removal of carbon, nitrogen, and/or sulfur under anoxic/anaerobic conditions have recently received considerable attention due to advantages such as cost and space reduction. Nitrate is released by various industrial and agricultural activities. Biological nitrate reduction has been successfully used for the removal of nitrate from wastewater. Nitrate reduction occurs in two distinct pathways: dissimilatory nitrate reduction to nitrogen gas (denitrification) and dissimilatory nitrate reduction to ammonia (DNRA). Sulfide, a common constituent in anaerobic digesters, plays an important role in the prevailing pathway of nitrate reduction. It has been reported that nitrate and/or reduced N-oxides, such as nitrite (NO_2^-), nitric oxide (NO), and nitrous oxide (N_2O), suppress methanogenesis. To date, research dealing with the effect of nitrate reduction on methanogenesis has been conducted either with pure cultures or with soil microcosms. Research involving anaerobic digestion with mixed methanogenic cultures has only investigated the effect of nitrate. In addition, a systematic study on the effect of nitrate and sulfide in the presence of different electron donors on the inhibition of methanogenesis is lacking.

Research presented here was conducted to investigate the effect of N-oxides and sulfide on a mixed methanogenic culture, along with the effect of the type of electron donor on the kinetics and pathway of nitrate reduction. The inhibitory effects of nitrate, NO_2^-, NO, and N_2O on a sulfide-free mixed, mesophilic (35^oC) methanogenic culture were investigated. Among all N-oxides, NO exerted the most and nitrate exerted the least inhibitory effect on the fermentative/methanogenic consortia. Long-term exposure of the

methanogenic culture to nitrate resulted in an increase of N-oxide reduction rates and decrease of methane production rates, which was attributed to changes in the microbial community. The effect of sulfide on nitrate reduction and methanogenesis was investigated in sulfide-free and sulfide-acclimated methanogenic cultures. Sulfide addition to sulfide-free enriched cultures resulted in inhibition of NO_2^-, NO, and N_2O reduction causing accumulation of these intermediates, which in turn inhibited methanogenesis and fermentation. In the nitrate-amended, sulfide-acclimated cultures, instead of accumulation of N-oxides, nitrate reduction occurred via DNRA and converted NO_2^- to ammonia; thus, accumulation of N-oxides was avoided and inhibition of methanogenesis was prevented. The effect of different electron donors on the pathway and kinetics of nitrate reduction was investigated in a sulfide-acclimated methanogenic culture. The nitrate reduction rates in the cultures fed with different substrates were as follows in descending order: H_2/CO_2 > acetate > glucose > dextrin/peptone > propionate. Denitrification was the dominant pathway of nitrate reduction in the propionate-, acetate-, and H_2/CO_2-fed cultures regardless of the COD/N ratio value. However, both denitrification and DNRA were observed in the dextrin/peptone- and glucose-fed cultures and the predominance of either of the two pathways was a function of the COD/N ratio value. Nitrate reduction processes were incorporated into the IWA Anaerobic Digestion Model No. 1 (ADM1) in order to account for the effect of nitrate reduction processes on fermentation and methanogenesis. The extended ADM1 described the experimental results very well. Model simulations showed that process interactions during nitrate reduction within an overall methanogenic system cannot be explained based on only stoichiometry and kinetics, especially for batch systems and/or continuous-flow systems

with periodic, shock nitrate loads. The results of this research are useful in predicting the fate of carbon-, nitrogen-, and sulfur-bearing waste material, as well as in understanding microbial process interactions, in both natural and engineered anoxic/anaerobic systems.

CHAPTER 1

INTRODUCTION

Anaerobic processes have many advantages over other biological unit operations, such as higher organic loading rates, lower sludge production, and energy production in the form of methane. Among these advantages, energy production has been a major driver for the observed increased application of anaerobic processes. In addition, combined treatment technologies for the removal of carbon, nitrogen, and/or sulfur under anoxic/anaerobic conditions have recently received considerable attention due to advantages such as cost and space reduction.

Nitrate is released by various industrial and agricultural activities, such as cellophane, pectin, and explosives production, metal finishing, and fertilizer applications. Biological nitrate reduction has been successfully used for the removal of nitrate from wastewater. Nitrate reduction occurs in two distinct pathways: dissimilatory nitrate reduction to nitrogen gas (denitrification) and dissimilatory nitrate reduction to ammonia (DNRA). Denitrification proceeds in stepwise manner in which nitrate (NO_3^-) is reduced to nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O), and nitrogen gas (N_2). DNRA is the production of ammonia by reduction of nitrate and/or nitrite, which occurs at very low redox potential values (Knowles, 1982), in the presence of sulfide (Brunet and Garcia-Gill, 1996) or at high COD/N ratios (Akunna et al., 1992), depending on the nature of the carbon source (Akunna et al., 1993). Denitrification prevailed against DNRA when volatile fatty acids (VFAs) were the main electron donor and DNRA

became the predominant nitrate reduction pathway when glycerol and glucose were used regardless of the COD/N ratio (Akunna et al., 1993). It has been reported that nitrate and/or reduced N-oxides, such as nitrite, nitric oxide, and nitrous oxide, suppress methane production (Akunna et al., 1994; Klüber and Conrad, 1998a; Klüber and Conrad, 1998b; Clarens et al., 1998; El-Mahrouki and Watson-Craik, 2004). Sulfide in nitrate reducing environments play an important role in the prevailing pathway of nitrate reduction (Myers, 1972; Brunet and Garcia-Gill, 1996; Percheron et al., 1998; Senga et al., 2006). Brunet and Garcia-Gill (1996) reported that at an extremely low free sulfide concentration (1.6 mg S/L), denitrification was the dominant nitrate reduction pathway, whereas at a relatively high free sulfide concentration (32 mg S/L), incomplete denitrification and ammonia production through DNRA was observed in freshwater sediments. At a high free sulfide concentration, nitrate reduction via DNRA took place because of partial inhibition of NO- and strong inhibition of N_2O-reductases by sulfide (Sørensen et al., 1980; Brunet and Garcia-Gill, 1996). Sulfide in anaerobic treatment systems are introduced via the influent wastewater, produced biologically via reduction of sulfate or other inorganic sulfur species, or as a result of degradation of sulfur-containing organic compounds (e.g., proteins).

The suppression of methanogenesis by N-oxides was first attributed to the increase in the redox potential due to nitrate addition. However, studies conducted under controlled redox conditions revealed that the suppression of methanogenesis was not related to changes in the redox potential (Balderston and Payne, 1976; Roy et al., 1997). Relative to the suppression of methanogenesis in the presence of N-oxides, two more mechanisms were proposed: substrate competition between nitrate reducers and

methanogens and inhibition of methanogens by N-oxides (Balderston and Payne, 1976). Inhibition of methanogens by N-oxides has been identified as the main mechanism involved in the suppression of methanogenesis (Roy and Conrad, 1999). The kinetics and/or preferential use of carbon/electron donor sources by nitrate reducers may be different in pure cultures compared to that of mixed cultures. In addition to competition, the denitrification intermediates are known to inhibit various bacterial species (Klüber and Conrad, 1998). Therefore, the accumulation of denitrification intermediates may result in the build up of these compounds and, thus, may cause inhibition of nitrate reduction and/or methanogenesis in mixed, overall methanogenic systems.

Relative to modeling and simulation of anaerobic treatment systems, several models of various complexities have been developed and used in the past (Pavlostathis and Giraldo-Gomez, 1991; Siegrist et al., 2002). The recently developed IWA Anaerobic Digestion Model No. 1 (ADM1) (Batstone et al., 2002) is a structured model, which includes multiple steps describing biochemical and physicochemical processes encountered in the anaerobic biodegradation of complex organic compounds. However, the ADM1 does not account for alternative electron accepting processes, such as nitrate reduction.

The main focus of the research presented here was to investigate the effect of N-oxides and sulfide, along with the effect of the type of electron donor on the kinetics and pathway of nitrate reduction in a mixed, overall methanogenic system. The research commenced with the assessment of the inhibitory effects of nitrogen oxides on a sulfide-free mixed methanogenic culture (Chapter 4). The effect of nitrate, nitrite, nitric oxide, and nitrous oxide on methanogenesis was assessed as a function of initial N-oxide

concentration. Then, the effect of sulfide on the nitrate reduction pathway and methanogenesis was investigated (Chapter 5). Abiotic assays were also performed to assess the possibility of nitrate reduction in the absence of microbial activity (Chapters 4 and 5). Preferential carbon/electron donor source utilization and the kinetics of nitrate reduction in a mixed, sulfide-enriched methanogenic culture were investigated (Chapter 6). Five types of carbon/electron donor sources were used: a mixture of dextrin/peptone, glucose, propionate, acetate, and H_2/CO_2. Finally process interactions of methanogenesis and nitrate reduction were modeled using ADM1 as a baseline model (Chapter 7). The ADM1 model was modified in terms of disintegration and hydrolysis processes and extended by the incorporation of nitrate reduction processes.

This research provides a better understanding of the process interactions of denitrification and methanogenesis, based on both the experimental results obtained in this study, as wells the development of a mathematical model. The extended ADM1 model is the first attempt to incorporate nitrate reduction processes into an anaerobic digestion model. The results of this research are applicable to both natural and engineered systems, in which process interactions among nitrate reducers and methanogens may be of concern. The research provided here will provide information on predicting the fate of carbon-, nitrogen-, and sulfur-bearing waste material, as well as in understanding microbial process interactions in both natural and engineered anoxic/anaerobic systems.

CHAPTER 2

BACKGROUND

2.1. Anaerobic Processes

Anaerobic processes such as methanogenesis, denitrification, sulfate reduction etc., which are used primarily for the treatment of high-strength organic wastes, have received considerable attention over the past ten years (McCarty, 2001). Anaerobic fermentation processes are advantageous because of the lower biomass yields and also because energy, in the form of methane, can be obtained from the biological conversion of organic substrates (Metcalf and Eddy, 2003).

The anaerobic degradation of complex, particulate organic matter has been described as a multi-step process of series and parallel reactions (Figure 2.1). First, complex polymeric materials, such as polysaccharides, proteins and lipids, are hydrolyzed. The resulting, relatively simple, soluble compounds (monomers) are fermented or anaerobically oxidized to short-chain fatty acids, alcohols, carbon dioxide, hydrogen and ammonia. The short chain fatty acids are converted to acetate, hydrogen gas, and carbon dioxide. Lastly, methane is formed by acetoclastic methanogenesis (i.e., the splitting of acetate to methane and carbon dioxide) and hydrogenotrophic methanogenesis (i.e., the reduction of carbon dioxide using hydrogen as the reductant) (Pavlostathis and Giraldo-Gomez, 1991). Bacteria, which carry out anaerobic respiration

generally possesses electron transport systems containing cytochromes, quinones, iron-sulfur proteins and other typical electron transport proteins (Madigan et al., 2005).

2.1.1. Fermentation and Methanogenesis

Anaerobic treatment is a natural process in which a variety of different species from two entirely different biological kingdoms, Bacteria and Archaea, work together to convert organic wastes through a variety of intermediates into methane gas (McCarty, 2001). The consortia of microorganisms involved in the overall conversion of complex organic matter to one-carbon compounds representing the most oxidized (CO_2) and most reduced (CH_4) begins with the bacteria that hydrolyze complex organic matter (carbohydrates, proteins and fats) into simple monomers, such as carbohydrates, amino acids and fatty acids (Rittmann and McCarty, 2001). The monomers are then utilized by fermenting bacteria producing organic acids and molecular hydrogen as the dominant intermediate products. The organic acids are then partially oxidized by other fermenting bacteria producing additional hydrogen and acetic acid which then are used by methanogens and converted to methane (Figure 2.1) (Rittmann and McCarty, 2001).

The term fermentation was first used by Pasteur to define respiration in the absence of free molecular oxygen. Fermentation is a form of respiration performed by facultative anaerobes and strict anaerobes, which involves transformation of organic compounds to various organic and inorganic compounds (Gerardi, 2003). During fermentation, a portion of the organic compound gets oxidized while the other portion is reduced. Through these oxidation-reduction reactions, fermentative bacteria obtain energy and produce various compounds with different oxidation states. Fermentation of

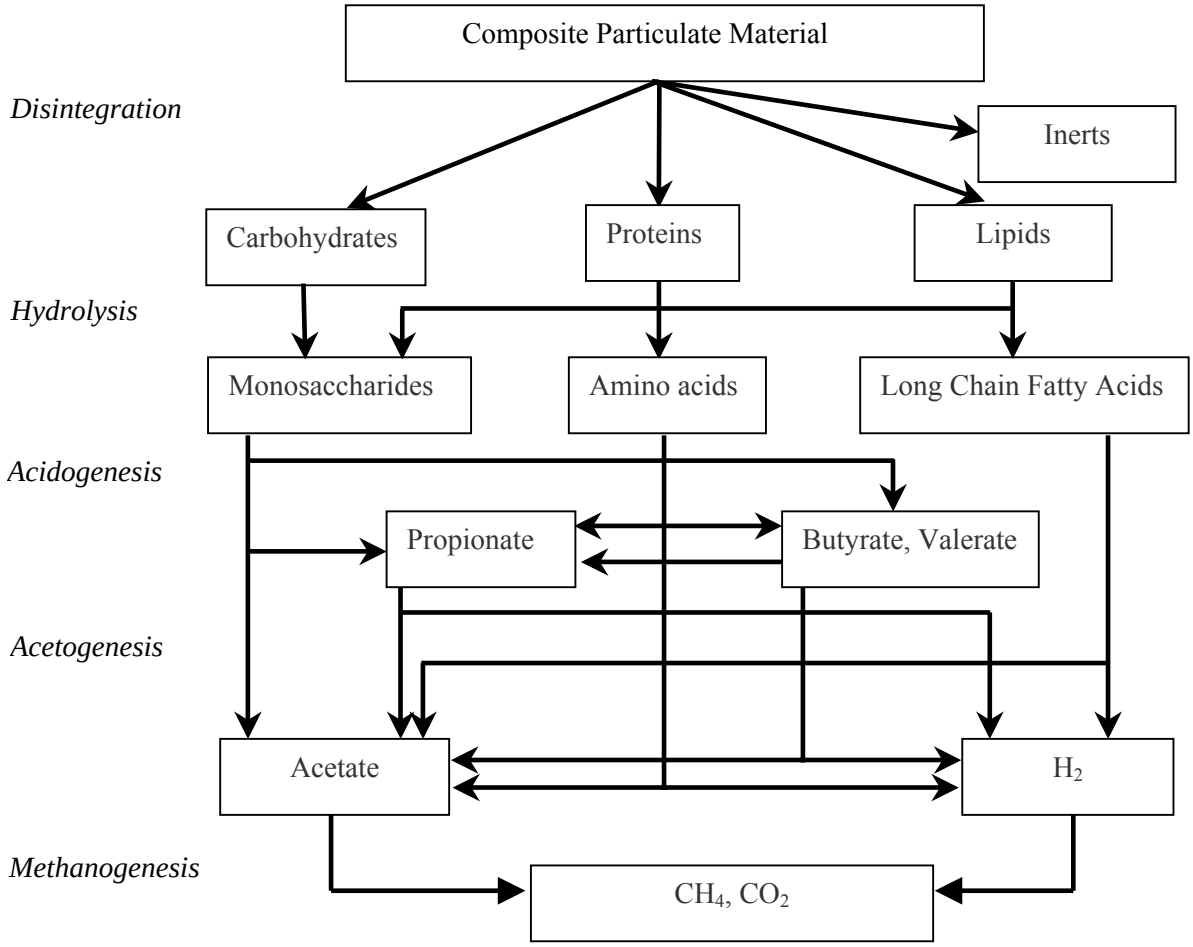

Figure 2.1. Carbon flow in typical anaerobic degradation processes (adopted from Batstone et al., 2002)

soluble carbohydrates results in the production of ethanol, acetate, H_2 and CO_2 in the absence of methanogenic bacteria (Pavlostathis and Giraldo-Gomez, 1991). The major end products of fermentation of amino acids are short-chain fatty acids, succinate, aminovalerate, and hydrogen gas (Pavlostathis and Giraldo-Gomez, 1991).

The biological production of methane is carried out by a group of strictly anaerobic *Archaea*, called methanogens (Zinder, 1993). In natural anaerobic environments, methanogens catalyze the terminal step of organic matter degradation to CO_2 and CH_4. Methanogenesis takes place in the absence of oxygen or other electron acceptors. Addition or presence of other, energetically more favorable electron acceptors than CO_2, such as nitrate, sulfate, Mn (IV), Fe (III), etc., usually results in the reduction of these electron acceptors instead of methane production (Klüber and Conrad, 1998). Methanogens are phylogenetically diverse and they have the ability to utilize few simple compounds, such as single-carbon compounds such as $HCOO^-$ (formate), CO_2, CO; methyl substrates, such as methanol, methylamine, methylmercaptan, etc.; and acetyl substrates such as acetate (Ferry, 1993). Therefore, methanogens dependent on other microorganisms for the production of substrate(s). It is still not known why the methanogens cannot utilize more complex carbon sources such as propionate or glucose. However, one view is that methanogenesis requires a very complex and specialized metabolic system and methanogens cannot compete with fermenters for complex carbon sources (Zinder, 2003).

Methanogens require sulfur and iron. In addition, other trace metals, such as cobalt, nickel, zinc, tungsten, manganese, molybdenum, selenium, and boron are required as stimulatory compounds for methanogenesis (Speece, 1996). Sulfide is also a required

nutrient, which must be supplied to an anaerobic system at a concentration of 0.001 to 1 mg S/L in order to ensure process stability, optimum microbial growth, and methane production (Speece, 1996). However, sulfide is also toxic to methanogens, but the inhibitory sulfide levels highly depend on the methanogenic species, pH, temperature, and the type of substrate used (Speece, 1996). Methanogens are found in environments of variable salinity, from freshwater to hypersaline. However, methanogens require at least 1 mM Na^+ to function because the inwardly directed sodium motive force is involved in the bioenergetics of methanogenesis (Zinder, 2003). Methanogens are found in a wide range of temperatures from 2^{o}C to above 100^{o}C. Optimum pH for methanogens is around neutrality (Zinder, 2003). However, there are methanogens, which survive at extreme environments at pH values of 4.0 or below (e.g., the hydrogenotrophic methanogen *Methanobacterium*) (Williams and Crawford, 1985). The optimum pH range for methanogens usually lies from 6.5 to 8.2 (Speece, 1996). Methanogens are strictly anaerobicand require oxidation-reduction potential of at least –0.3V in order to survive, which theoretically corresponds to 10^{-56} mole O_2/L (Hungate, 1967).

In anaerobic digesters, sediments and soils about two thirds of the methane produced is from acetate and the rest from H_2/CO_2 (Boone, 1982; Lovley and Klug, 1982; Zinder et al., 1984). In marine sediments, H_2/CO_2 is identified as the main precursor for methanogenesis (Claypool and Kaplan, 1974). Methanogens compete with three other major metabolic groups for their substrates in natural habitats: sulfate reducing bacteria, acetogens, and iron reducers.

2.1.2. Nitrate Reduction

Nitrogen is a vital component in essential biomolecules, such as proteins, nucleic acids (i.e., DNA, RNA) and it is found in oxidation states from –III (NH_4^+) to +V (NO_3^-) in the biosphere. The inorganic nitrogen cycle is carried out by prokaryotes as shown in Figure 2.2. Nitrate contamination of both surface and ground water has become a problem in many parts of the world. Consumption of nitrate may cause methaemoglobinaemia in babies and also reduction of nitrates to nitrites in saliva may lead to the formation of nitrosamines, which are known carcinogens. Nitrate reducers are very widespread in nature (Knowles, 1982). Biological nitrogen removal is one of the most effective ways of removing nitrogen from water/wastewater (Chaudhry and Beg, 1997).

Nitrate reduction occurs in two distinct pathways: dissimilatory nitrate reduction to nitrogen gas (denitrification) and dissimilatory nitrate reduction to ammonia (DNRA). The capacity for nitrate reduction is present in strains contained in ten different prokaryotic families; only three groups of microorganisms do not have representatives that have the ability to reduce nitrogen oxides: (1) obligate anaerobes, (2) most of the gram-positive organisms, and (3) the *Enterobacteriacaeae* (Tiedje, 1988). The most common nitrate reducers in nature are species of *Pseudomonas* followed by closely related *Alcaligenes* (Tiedje, 1988). *Alcaligenes*, *Pseudomonas aeruginosa*, *Achromobacter*, and *Bacillus* are the most common denitrifying bacteria found in wastewater treatment plants (Table 2.1) (Knowles, 1982). Denitrification is mediated by heterotrophic bacteria, which use nitrate or other oxidized forms of nitrogen as electron acceptor and reduce them to nitrogen gas in the absence of oxygen (Tiedje, 1988; El-

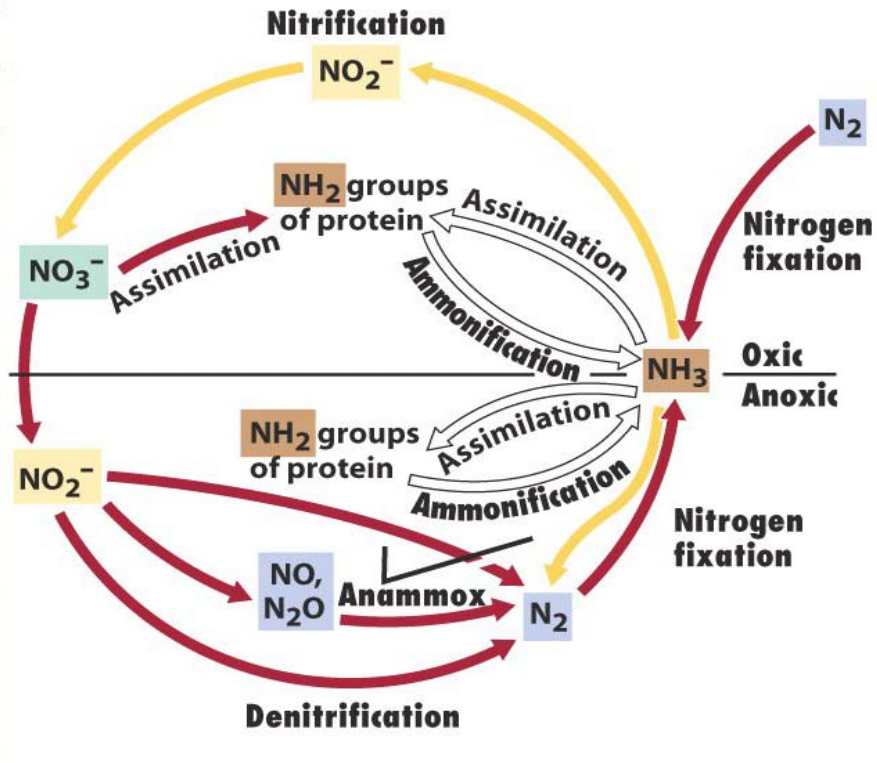

Figure 2.2. Redox cycle for nitrogen. Oxidation reactions are represented by yellow arrows and reductions in red. Reactions in which no redox changes occur are in white (Madigan et. al., 2005).

Mahrouki and Watson – Craik, 2004). A variety of facultative and obligate anaerobic species are capable of reducing nitrate to nitrite. However, the fate of nitrite produced varies from bacterial species to species. Some bacteria reduce nitrite no further, whereas some other bacteria reduce nitrite all the way down to nitrogen gas, or ammonia (Yordy and Ruoff, 1988).

2.1.2.1. Dissimilatory Nitrate Reduction to Nitrogen (Denitrification)

Denitrification is the reduction of oxidized nitrogen compounds like nitrate and nitrite to gaseous nitrogen compounds like nitrous oxide and nitrogen. Denitrifiers are mostly facultative bacteria, which use N-oxides in the absence of oxygen as an alternative electron acceptor (Tiedje, 1988). Denitrification is performed by chemoorganotrophic, lithoauthotrophic, phototrophic bacteria and some algae under anoxic/anaerobic conditions (Zumft, 1997). Although the majority of denitrifying bacteria are Gram-negative, denitrifying bacteria are also well represented among Gram-positive bacteria (Table 2.1). Denitrification can be described as an anaerobic respiration in which the electrons originated from organic or inorganic matter (e.g., sulfide or molecular hydrogen) are transferred to oxidized nitrogen compounds in order to create a proton motive force across the cytoplasmic membrane to generate ATP (Schmidt et al., 2003).

Denitrification proceeds in stepwise manner in which nitrate (NO_3^-), is reduced to nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O) and nitrogen gas (N_2) (Rittmann and McCarty, 2001). Denitrification may also occur in the presence of oxygen. The range of oxygen concentrations permitting aerobic denitrification differs from microorganism to microorganism. The onset of aerobic denitrification does not depend on the oxygen

Table 2.1. Nitrate reducing archaeal and bacterial genera (adopted from Zumft, 1997)

Archaea

Organotrophic

Halophilic

Haloarcula

Halobacterium

Halofenax

Hyperthermophilic

Pyrobacterium

Bacteria (Gram-positive)

Organotrophic

Spore Forming

Bacillus

Non-spore forming

Jonesia

Bacteria (Gram-negative)

Phototrophic

Rhodobacter

Rhodopseudomonas

Rhodoplanes

Lithotrophic

S oxidizing

Beggiatoa

Thiobacillus

Thioploca

H_2 Oxidizing

Ralstonia

Paracoccus

Pseudomonas

NO_2^- or NH_4^+ oxidizing

Nitrobacter

Nitrosomonas

Organotrophic

Carboxidotrophic

Pseudomonas

Zavarzinia

Oligocarbophilic

Aquasipirillum

Hyphomicrobium

Fermentative

Empedobacter

Azosipirillum

Faculatively Anaerobic

Alteromonas

Pseudomonas

Aerobic

Paracoccus

Alcaligenes

Diazotrophic

Aquasipirillum

Azospirillum

Azoarcus

Bacillus

Bradyrhizobium

Pseudomonas

Rhodobacter

Rhodopseudomonas

Sinorhizobium

Thermophilic

Aquifex

Bacillus

Thermothrix

Psychrophilic

Aquasipirillum

Halomonas

Halophilic

Halomonas

Bacillus

Pigment-forming

Chromobacterium

Flavobacterium

Budding

Blastobacter

Hyphomicrobium

Gliding

Cytophaga

Flexibacter

Magnetotactic

Magnetosipirillum

Pathogenic

Achromobacter

Alcaligenes

Agrobacterium

Campylobacter

Eikenella

Flavobacterium

Kingella

Moraxella

Morococcus

Neisseria

Ochrobactrum

Oligella

Pseudomonas

Sphingobacterium

Tsukamurella

sensitivity of the corresponding enzymes, but rather it depends on the regulation of oxygen redox sensing factors involved in the regulation on a transcriptional level (Robertson et al., 1990). The switch from aerobic conditions to anaerobic conditions results in an immediate decrease in the a-type cytochromes and an increase of c-type cytochromes. In general, electrons are transported by cytochrome b to nitrate, c- and d-type cytochromes to nitrite, a- and c-type cytochrome to nitric oxide, and b- and c-type cytochromes to nitrous oxide (Schulp and Stouthamer, 1970). Certain microorganisms possess the entire denitrification pathway and some of them are capable of catalyzing one or a few steps of the pathway (Ingraham, 1981). Partial denitrification occurs for several physiological or genetic reasons: (1) an intermediate of the pathway but not nitrate is available to the bacterium; (2) environmental conditions (O_2 concentration, pH, or concentration of an intermediate of the pathway) render one or more steps of the pathway nonfunctional; (3) owing to different rates of induction of the various N-oxide reductases, if only certain of these enzymes are present in the cells at various times following the onset of induction; and (4) certain bacteria are not genetically capable of synthesizing the complete array of nitrogen oxide reductases of the denitrification pathway.

Enzymes involved in the denitrification process are nitrate reductase (NAP), nitrite reductase (NIR), nitric oxide reductase (NOR), and nitrous oxide reductase (NOS) (Zumft et al., 1988). Some microorganisms possess all the reductases such as *Pseudomonas stutzeri* and are capable of reducing nitrate to nitrogen gas (Figure 2.3) However, some microorganisms are capable of only reducing nitrate to nitrite, and are lacking nitrite reductase, nitric oxide reductase, and nitrous oxide reductase (Ingraham, 1981). Escherichia coli is one of these organisms, which only carry out nitrate reduction

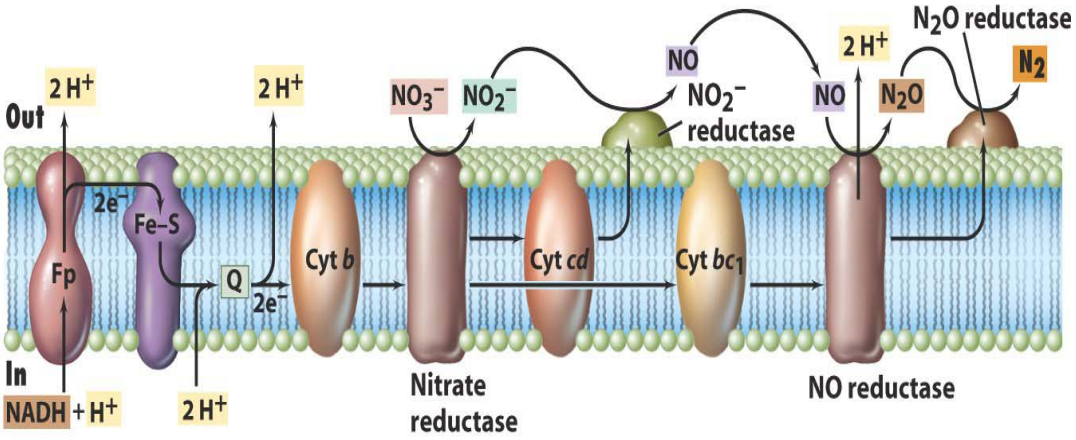

Figure 2.3. Possible scheme for electron transport in membranes of *Pseudomonas stutzeri* during denitrification (Madigan et. al., 2005).

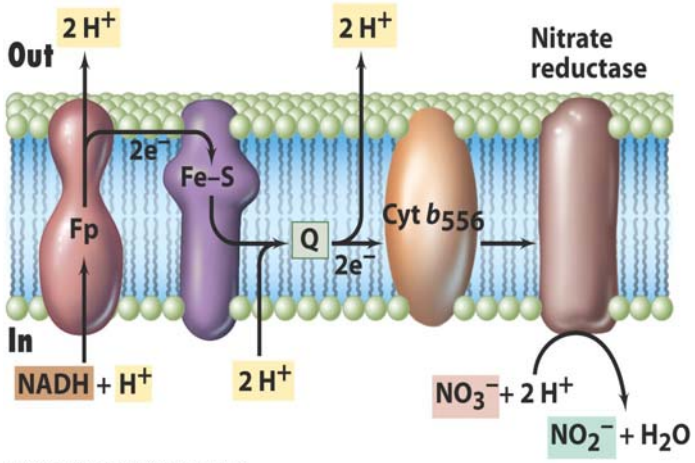

Figure 2.4. Electron transport processes in membrane of *Escherichia coli* when nitrate is used as an electron acceptor and NADH as electron donor (Madigan et. al., 2005).

to nitrite (Figure 2.4). The organisms that are capable of converting nitrate to nitrite are called to carry out nitrate respiration and are the most numerous of all the denitrifiers (Table 2.2). Certain types of organisms are capable of reducing nitrate to nitrous oxide and generally lack the nitrous oxide reductase gene (e.g., *Aquaspirillum itersonii* and some strains of *Pseudomonas fluorescens*). Some organisms do not possess nitrate reductase, thus, they can only reduce nitrite to nitrogen gas (e.g., species of *Neisseria*). Some organisms do not possess nitrite reductase (e.g., *Bacillus licheniformis*), therefore, they have to be in close proximity to other bacteria possessing nitrite reductase in order to carry out complete denitrification (Ingraham, 1981). Nitrate reductases have been classified by taking into consideration source, localization of the enzyme in the cell, molecular properties of the catalytic center, and function (Gonzales et al., 2006). All of them are mononuclear Mo-containing enzymes that belong to the dimethyl sulfoxide (DMSO) reductase family. There are three types of nitrate reductases, soluble assimilatory reductase and two dissimilatory reductases subdivided into membrane-bound respiratory and periplasmic nitrate reductases (Zumft, 1997). Membrane-bound respiratory nitrate reductases are expressed only under anaerobic growth conditions, whereas, periplasmic nitrate reductases are synthesized and active in the presence of oxygen (Bell et al., 1990). For the reduction of nitrite, there are two entirely different enzymes in terms of structure and the prosthetic metal groups (Zumft, 1997). However, these nitrite reductases have not been found in the same cell yet. About three quarters of strains collected worldwide have the tetraheme protein cytochrome cd1 as the respiratory nitrite reductase and the rest have copper-containing nitrite reductase (Gamble et al., 1977). The structures of these enzymes are known, except for nitric oxide reductase

Table 2.2. Microorganisms capable of reducing nitrate to nitrite (nitrate respiration)[a].

Actinobacillus	*Escherichia*	*Peptococcus*
Actinomyces	*Eubacterium*	*Photobacterium*
Aeromonas	*Flavobacterium*	*Planomonospora*
Agrobacterium	*Fusobacterium*	*Plesiomonas*
Alcaligenes	*Geodermatophilus*	*Propionibacterium*
Arachnia	*Haemophilus*	*Proteus*
Arthrobacter	*Halobacterium*	*Pseudomonas*
Bacillus	*Halococcus*	*Rhizobium*
Bacterionema	*Hyphomicrobium*	*Rothia*
Bacteroides	*Hyphomonas*	*Salmonella*
Beneckea	*Klebsiella*	*Selenomonas*
Bordetella	*Lactobacillus*	*Serratia*
Branhamella	*Leptothrix*	*Shigella*
Brucella	*Listeria*	*Simonsiella*
Campylobacter	*Lucibacterium*	*Spirillum*
Cellulomonas	*Microbispora*	*Sporosarcina*
Chromobacterium	*Micrococcus*	*Streptomyces*
Citrobacter	*Micromonospora*	*Streptosporangium*
Clostridium	*Moraxella*	*Thiobacillus*
Corynebacterium	*Mycobacterium*	*Thiomicrospira*
Cytophaga	*Neisseria*	*Veillonella*
Dactylsporangium	*Nocardia*	*Vibrio*
Enterobacter	*Paracoccus*	
Erwinia	*Paseurella*	

[a] Source: (Tiedje, 1988)

(Moura et al., 2003). The respiratory nitrate reductase is a very simple enzyme containing a single subunit with one [4Fe-4S] and a molybdenum cofactor, which is the active site of the enzyme. NIR has five heme groups and two Ca^{2+} sites. NOR contains one binuclear and tetranuclear copper center (Moura et al., 2003).

Although, denitrifiers require an electron donor to survive, upon finding denitrifying bacteria in nitrate- or oxygen-free environments, it has been shown that nitrate reducers can survive in anaerobic environments by carrying on a low level of fermentation at a rate sufficient for their survival (Jørgensen and Tiedje, 1993). Denitrifiers are very versatile in terms of energy source. In fact, the energy sources of nitrate reducers include all three classes known to be used by microorganisms: organic (organotrophs), inorganic (lithotrophs), and light (phototrophs) (Tiedje, 1988).

Copper is required for denitrification, because some organisms have copper-containing protein as a nitrite reductase; other organisms have cytochrome-cd type nitrite reductase, which does not contain copper but requires copper for its synthesis (Bryan, 1981). Iron is necessary for activity of the denitrification enzymes because it is found in both the heme and non-heme groups of these enzymes. Sulfur is also required for enzyme activity. Molybdenum is the integral part of all denitrification enzymes and magnesium is required for the growth of denitrifiers.

Optimum pH for denitrification varies from microorganism to microorganism; however, it is generally in the range if 5.8 to 9.2 (Bryan, 1981). pH also has an effect on the distribution of gases of denitrification, at pH 7.0 and below N_2O is the major

denitrification product, whereas, above pH 7.0, N_2O is produced but subsequently utilized and converted to N_2 (Delwiche, 1954).

2.1.2.2. Assimilatory and Dissimilatory Nitrate Reduction to Ammonia (DNRA)

The reduction of nitrate or nitrite to ammonia is classically identified as *assimilatory* nitrate reduction. The assimilatory process involves the conversion of nitrate and/or nitrite to ammonia, which is used by the cell to incorporate nitrogen into biomolecules (Richardson and Watmough, 1999).

DNRA is an anaerobic process. Oxygen inhibits the activity of the enzymes involved in DNRA, which results from the oxidation of the dissimilatory nitrate reductase (Stouthamer, 1976). Conservation of energy in the form of ATP seems to be the primary function of dissimilatory nitrate reduction to nitrite. Facultative anaerobic bacteria possessing this capacity can carry out electron-transport-coupled phosphorylation (ETP) under anaerobic conditions when nitrate is available, as well as under aerobic conditions, which gives metabolic flexibility to these bacterial species (Yordy and Ruoff, 1988). For strict anaerobes, dissimilatory nitrate reduction provides a means of ATP production that supplements the usual substrate-level phosphorylation. The reduction of nitrate or nitrite to ammonia is classically identified as assimilatory nitrate reduction as mentioned above (Yordy and Ruoff, 1988). The nitrate reductase involved in the DNRA process is different than the one used in the assimilatory nitrate reduction to ammonia. The dissimilatory nitrate reductase is a membrane-bound protein, which can only function under anaerobic conditions (Boxer and Clegg, 1975). The enzyme responsible for the physiological reduction of nitrite to ammonia is cytoplasmic, requires NADH, and also catalyzes the reduction of hydroxylamine (Yordy and Ruoff, 1988). Many bacteria that

are capable of reducing nitrite to ammonia possess more than one nitrite reductase, which complicates research in this area (Yordy and Ruoff, 1988).

The DNRA process starts with the reduction of nitrate to nitrite, which is then reduced to ammonia (Gonzales et al., 2006). The DNRA process is very favorable in terms of energy production because the reduction of nitrate to ammonium accommodates eight electrons per nitrogen. DNRA has been found in bacteria that have fermentative rather than oxidative metabolisms, which is the opposite of denitrification (Samuelson, 1985). The capacity to dissimilate nitrate to ammonium is common among anaerobic microorganisms (Table 2.3). DNRA has also been observed in microaerophilic and some aerobic microorganisms, however such information is very limited. The rate-limiting step of the DNRA process is the reduction of nitrite to ammonium (Tiedje, 1988). The cell benefits from the DNRA process in many ways. DNRA is used (a) to detoxify the accumulated nitrite, (b) serves as an electron sink, which allows the reoxidation of NADH, and (c) produces energy through electron transport phosphorylation (ETP). The main energy-producing step of DNRA is the reduction of nitrate to nitrite. During the reduction of nitrite to ammonium, soluble nitrite reductase is used, which prevents conservation of energy. Therefore, nitrite accumulates and is not converted to ammonium under carbon-limited conditions (Tiedje, 1988). Under nitrate-limiting conditions, the need for an electron sink is more important and thus nitrite is converted to ammonium. Some of the soluble nitrite reductases are NADH-linked and one of the enzymes is a sulfite reductase. Therefore, in the presence of sulfide, nitrite reduction to ammonium may be enhanced because it is an alternative substrate for the sulfite reduction pathway (Coleman et al., 1978). DNRA activity is high in carbon rich, electron acceptor poor

Table 2.3. Microorganisms capable of DNRA (adopted from Tiedje, 1988)

Organism	Habitat
Obligate anaerobes	
Clostridium spp.	Soil, sediments
Veillonella alcalescens	Intestinal tract
Wolinella (Vibrio) succinogens	Rumen
Desulfovibrio desulfuricans	Sediment
Desulfovibrio gigas	Sediment
Desulfovibrio species	Sediment
Selenomonas ruminantium	Rumen
Facultative	
Escherichia coli	Soil, wastewater
Citrobacter spp.	Soil, wastewater
Salmonella typhimurium	Sewage
Klebsiella spp.	Soil, wastewater
Enterobacter (Aerobacter) aerogenes	Soil, wastewater
Serratia marcescens	
Erwinia carotovora	Soil
Photobacterium (*Achromobacter*) fischeri	Sea
Vibrio (several species)	Sediment
Microaerophile	
Campylobacter sputorum	Oral cavity
Aerobes	
Pseudomonas (several strains)	Soil, water
Neisseria subflava	Mucous membranes
Bacillus (several strains)	Soil, food

environments. Therefore, the anaerobic environments are best for DNRA populations. DNRA becomes the main nitrate and nitrite reduction pathway at COD/N values greater than 53 (Akunna, 1992). Presence of sulfide also causes the prevalence of DNRA (Myers, 1992; Brunet and Garcia-Gill, 1996). All of the microorganisms carrying out DNRA are able to couple H_2, or formate oxidation to ATP production via DNRA (Brunet and Garcia-Gill, 1996). In addition, *Desulfovibrio desulfuricans* and *Desulfobulbus propionicus* are able to couple nitrate and nitrite ammonification to the oxidation of inorganic sulfur compounds (Dannenberg et al., 1992).

Several physiological electron donors for DNRA have been identified, and include organic compounds such as glucose, pyruvate, lactate, glycerol, and formate, as well as inorganic electron donors such as molecular hydrogen (Yordy and Ruoff, 1988).

2.2. Process Interactions Involving Fermentation, Methanogenesis and Nitrate Reduction

Nitrate reducers, fermenters, and methanogens coexist in the environment due to the widespread occurrence of nitrate reducers. This coexistence may cause two groups of microorganisms to compete for the same substrate(s) due to the wide variety of carbon sources utilized by nitrate reducers (Roy and Conrad, 1999; Batstone et al., 2002). As mentioned before, nitrate reducers have the ability to utilize a variety of fermentative/methanogenic substrates such as glucose, glutamic acid (Marazioti et al.,2003), VFAs (Aboutboul and Rijn, 1995; Elefsiniotis et al., 2004), hydrogen (Scheid et al., 2003), and even methane (Eisentraeger, 2001). Among other VFAs, the propionic acid has been reported as the most preferred carbon source by nitrate reducers

(Aboutboul and Rijn, 1995; Elefsiniotis et al., 2004). In addition, it has been shown that addition of nitrate, nitrite, and N_2O to a mixed fermentative/methanogenic culture resulted in a decrease of H_2 partial pressures to a level, which no longer allowed methanogenesis (Allison and Macfarlane, 1988; Klüber and Conrad, 1998b; Scheid et al., 2003).

Stoichiometric equations can be used to drive mole relationship between products and reactants for a particular process (Rittmann and McCarty, 2001). Rittmann and McCarty (2001) describe the derivation of stoichiometric equations based on thermodynamic and bioenergetic principles, which allow the estimation of microbial yield coefficients and specific substrate utilization rates. Stoichiometric equations pertinent to this study were derived based on the methodology described by Rittmann and McCarty (2001) and are reported below and summarized in Table 2.4. For batch systems used in the present study, the effect of solids retention time (SRT) and the microbial decay rate were taken into account in the derivation of these stoichiometric equations. As shown in Table 2.4, as a result of higher energy yield during denitrification, the bacterial yield is higher as compared to that for methanogenesis. In addition, stoichiometrically, complete nitrate reduction to nitrogen gas requires 5 electron equivalents per mol of nitrate. Therefore, coexistence of fermenters/methanogens and denitrifiers may cause channeling of electrons away from methanogenesis, which may result in a decrease in the overall methane production (Batstone et al., 2002). A select number of stoichiometric equations are provided below for various electron donors.

Denitrification with glucose:

$$\frac{1}{24}C_6H_{12}O_6 + \frac{1}{4}H_2O \rightarrow \frac{1}{4}CO_2 + H^+ + e^- \qquad \Delta G^{0'} = -41.35 \text{ kJ/eeq}$$

$$\frac{1}{5}NO_3^- + \frac{6}{5}H^+ + e^- \rightarrow \frac{1}{10}N_2 + \frac{3}{5}H_2O \qquad \Delta G^{0'} = -72.20 \text{ kJ/eeq}$$

Sum:

$$\frac{1}{24}C_6H_{12}O_6 + \frac{1}{5}NO_3^- + \frac{1}{5}H^+ \rightarrow \frac{7}{20}H_2O + \frac{1}{4}CO_2 + \frac{1}{10}N_2$$

$$5C_6H_{12}O_6 + 24NO_3^- + 24H^+ \rightarrow 12N_2 + 30CO_2 + 42H_2O$$

$\Delta G^{0'}$ =-2720 kJ/mol glucose = -113.3 kJ/eeq

Denitrification with propionate:

$$\frac{1}{14}CH_3CH_2COO^- + \frac{5}{14}H_2O \rightarrow \frac{1}{7}CO_2 + \frac{1}{14}HCO_3^- + H^+ + e^- \qquad \Delta G^{0'} = -27.63 \text{ kJ/eeq}$$

$$\frac{1}{5}NO_3^- + \frac{6}{5}H^+ + e^- \rightarrow \frac{1}{10}N_2 + \frac{3}{5}H_2O \qquad \Delta G^{0'} = -72.20 \text{ kJ/eeq}$$

Sum:

$$\frac{1}{14}CH_3CH_2COO^- + \frac{1}{5}NO_3^- + \frac{1}{5}H^+ \rightarrow \frac{17}{70}H_2O + \frac{1}{7}CO_2 + \frac{1}{14}HCO_3^- + \frac{1}{10}N_2$$

$$5CH_3CH_2COO^- + 14NO_3^- + 14H^+ \rightarrow 17H_2O + 10CO_2 + 5HCO_3^- + 7N_2$$

$\Delta G^{0'}$ = -1397.7 kJ/mol propionate = -99.83 kJ/eeq

Denitrification with acetate:

$$\frac{1}{8}CH_3COO^- + \frac{3}{8}H_2O \rightarrow \frac{1}{8}CO_2 + \frac{1}{8}HCO_3^- + H^+ + e^- \qquad \Delta G^{0'} = -27.4 \text{ kJ/eeq}$$

$$\frac{1}{5}NO_3^- + \frac{6}{5}H^+ + e^- \rightarrow \frac{1}{10}N_2 + \frac{3}{5}H_2O \qquad \Delta G^{0'} = -72.20 \text{ kJ/eeq}$$

Sum:

$$\frac{1}{8}CH_3COO^- + \frac{1}{5}NO_3^- + \frac{1}{5}H^+ \rightarrow \frac{9}{40}H_2O + \frac{1}{8}CO_2 + \frac{1}{8}HCO_3^- + \frac{1}{10}N_2$$

$$5CH_3COO^- + 8NO_3^- + 8H^+ \rightarrow 9H_2O + 5CO_2 + 5HCO_3^- + 4N_2$$

$\Delta G^{0'}$ = -796.8 kJ/mol acetate = -99.6 kJ/eeq

Denitrification with H_2:

$$\frac{1}{2}H_2 \rightarrow H^+ + e^- \qquad \Delta G^{0'} = -39.87 \text{ kJ/eeq}$$

$$\frac{1}{5}NO_3^- + \frac{6}{5}H^+ + e^- \rightarrow \frac{1}{10}N_2 + \frac{3}{5}H_2O \qquad \Delta G^{0'} = -72.20 \text{ kJ/eeq}$$

Sum:

$$\frac{1}{2}H_2 + \frac{1}{5}NO_3^- + \frac{1}{5}H^+ \rightarrow \frac{3}{5}H_2O + \frac{1}{10}N_2$$

$$5H_2 + 2NO_3^- + 2H^+ \rightarrow 6H_2O + N_2$$

$\Delta G^{0'}$ = -224.14 kJ/mol H_2 = -112.07 kJ/eeq

DNRA with glucose:

$$\frac{1}{24}C_6H_{12}O_6 + \frac{1}{4}H_2O \rightarrow \frac{1}{4}CO_2 + H^+ + e^- \qquad \Delta G^{0'} = -41.35 \text{ kJ/eeq}$$

$$\frac{1}{8}NO_3^- + \frac{5}{4}H^+ + e^- \rightarrow \frac{1}{8}NH_4^+ + \frac{3}{8}H_2O \qquad \Delta G^{0'} = -35.11 \text{ kJ/eeq}$$

Sum:

$$\frac{1}{24}C_6H_{12}O_6 + \frac{1}{8}NO_3^- + \frac{1}{4}H^+ \rightarrow \frac{1}{8}H_2O + \frac{1}{4}CO_2 + \frac{1}{8}NH_4^+$$

$$8\,C_6H_{12}O_6 + 24NO_3^- + 48H^+ \rightarrow 12NH_4^+ + 48CO_2 + 12H_2O$$

$\Delta G^{0'}$ =-1835.04 kJ/mol glucose = -76.46 kJ/eeq

DNRA with propionate:

$$\frac{1}{14}CH_3CH_2COO^- + \frac{5}{14}H_2O \rightarrow \frac{1}{7}CO_2 + \frac{1}{14}HCO_3^- + H^+ + e^- \qquad \Delta G^{0'} = -27.63 \text{ kJ/eeq}$$

$$\frac{1}{8}NO_3^- + \frac{5}{4}H^+ + e^- \rightarrow \frac{1}{8}NH_4^+ + \frac{3}{8}H_2O \qquad \Delta G^{0'} = -35.11 \text{ kJ/eeq}$$

Sum:

$$\frac{1}{14}CH_3CH_2COO^- + \frac{1}{8}NO_3^- + \frac{1}{4}H^+ \rightarrow \frac{1}{56}H_2O + \frac{1}{7}CO_2 + \frac{1}{14}HCO_3^- + \frac{1}{8}NH_4^+$$

$$8CH_3CH_2COO^- + 14NO_3^- + 28H^+ \rightarrow 2H_2O + 16CO_2 + 8HCO_3^- + 14NH_4^+$$

$\Delta G^{0'}$ = -878.4 kJ/mol propionate = -62.74 kJ/eeq

DNRA with acetate:

$$\frac{1}{8}CH_3COO^- + \frac{3}{8}H_2O \rightarrow \frac{1}{8}CO_2 + \frac{1}{8}HCO_3^- + H^+ + e^- \qquad \Delta G^{0'} = -27.4 \text{ kJ/eeq}$$

$$\frac{1}{8}NO_3^- + \frac{5}{4}H^+ + e^- \rightarrow \frac{1}{8}NH_4^+ + \frac{3}{8}H_2O \qquad \Delta G^{0'} = -35.11 \text{ kJ/eeq}$$

Sum:

$$\frac{1}{8}CH_3COO^- + \frac{1}{8}NO_3^- + \frac{1}{4}H^+ \rightarrow \frac{1}{8}CO_2 + \frac{1}{8}HCO_3^- + \frac{1}{8}NH_4^+$$

$$CH_3COO^- + NO_3^- + 2H^+ \rightarrow CO_2 + HCO_3^- + NH_4^+$$

$\Delta G^{0'}$ = -500 kJ/mol acetate = -62.5 kJ/eeq

DNRA with H_2:

$$\frac{1}{2}H_2 \rightarrow H^+ + e^- \qquad \Delta G^{0'} = -39.87 \text{ kJ/eeq}$$

$$\frac{1}{8}NO_3^- + \frac{5}{4}H^+ + e^- \rightarrow \frac{1}{8}NH_4^+ + \frac{3}{8}H_2O \qquad \Delta G^{0'} = -35.11 \text{ kJ/eeq}$$

Sum:

$$\frac{1}{2}H_2 + \frac{1}{8}NO_3^- + \frac{1}{4}H^+ \rightarrow \frac{3}{8}H_2O + \frac{1}{8}NH_4^+$$

$$4H_2 + 2NO_3^- + 4H^+ \rightarrow 6H_2O + 2NH_4^+$$

$\Delta G^{0'}$ = -150 kJ/mol H_2 = -74.98 kJ/eeq

Methanogenesis with glucose:

$$\frac{1}{24}C_6H_{12}O_6 + \frac{1}{4}H_2O \rightarrow \frac{1}{4}CO_2 + H^+ + e^- \qquad \Delta G^{0'} = -41.35 \text{ kJ/eeq}$$

$$\frac{1}{8}CO_2 + H^+ + e^- \rightarrow \frac{1}{8}CH_4 + \frac{1}{4}H_2O \qquad \Delta G^{0'} = 23.53 \text{ kJ/eeq}$$

Sum:

$$\frac{1}{24}C_6H_{12}O_6 \rightarrow + \frac{1}{8}CH_4 + \frac{1}{8}CO_2$$

$$C_6H_{12}O_6 \rightarrow 3CH_4 + 3CO_2$$

$\Delta G^{0'}$ = = -427.7 kJ/mol propionate = -17.82 kJ/eeq

Methanogenesis with propionate:

$$\frac{1}{14}CH_3CH_2COO^- + \frac{5}{14}H_2O \rightarrow \frac{1}{7}CO_2 + \frac{1}{14}HCO_3^- + H^+ + e^- \qquad \Delta G^{0'} = -27.63 \text{ kJ/eeq}$$

$$\frac{1}{8}CO_2 + H^+ + e^- \rightarrow \frac{1}{8}CH_4 + \frac{1}{4}H_2O \qquad \Delta G^{0'} = 23.53 \text{ kJ/eeq}$$

Sum:

$$\frac{1}{14}CH_3CH_2COO^- + \frac{3}{28}H_2O \rightarrow \frac{1}{8}CH_4 + \frac{1}{56}CO_2 + \frac{1}{14}HCO_3^-$$

$$4CH_3CH_2COO^- + 6H_2O \rightarrow 7CH_4 + CO_2 + 4HCO_3^-$$

$\Delta G^{0'}$ = = -57.4 kJ/mol propionate = -4.1 kJ/eeq

Methanogenesis with acetate:

$$\frac{1}{8}CH_3COO^- + \frac{3}{8}H_2O \rightarrow \frac{1}{8}CO_2 + \frac{1}{8}HCO_3^- + H^+ + e^- \qquad \Delta G^{0'} = -27.4 \text{ kJ/eeq}$$

$$\frac{1}{8}CO_2 + H^+ + e^- \rightarrow \frac{1}{8}CH_4 + \frac{1}{4}H_2O \qquad \Delta G^{0'} = 23.53 \text{ kJ/eeq}$$

Sum:

$$\frac{1}{8}CH_3COO^- + \frac{1}{8}H_2O \rightarrow \frac{1}{8}CH_4 + \frac{1}{8}HCO_3^-$$

$$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$$

$\Delta G^{0'}$ = -30.96 kJ/mol acetate = -3.87 kJ/eeq

Methanogenesis with H_2:

$$\frac{1}{2}H_2 \rightarrow H^+ + e^- \qquad \Delta G^{0'} = -39.87 \text{ kJ/eeq}$$

$$\frac{1}{8}CO_2 + H^+ + e^- \rightarrow \frac{1}{8}CH_4 + \frac{1}{4}H_2O \qquad \Delta G^{0'} = 23.53 \text{ kJ/eeq}$$

Sum: $\frac{1}{2}H_2 + \frac{1}{8}CO_2 \rightarrow \frac{1}{8}CH_4 + \frac{1}{4}H_2O$

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$$

$\Delta G^{0'}$ = = -32.68 kJ/mol H_2 = -16.34 kJ/eeq

Table 2.4. Gibbs free energy values for nitrate reduction and methanogenesis with various electron donors.

	$\Delta G^{0'}$ (kJ/mol ed)	$\Delta G^{0'}$ (kJ/eeq)
METHANOGENESIS		
$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	-33	-16
$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	-31	-4
$4CH_3CH_2COO^- + 6H_2O \rightarrow 7CH_4 + CO_2 + 4HCO_3^-$	-57	-4
$C_6H_{12}O_6 \rightarrow 3CH_4 + 3CO_2$	-428	-18
DENITRIFICATION		
$5H_2 + 2NO_3^- + 2H^+ \rightarrow 6H_2O + N_2$	-224	-112
$5CH_3COO^- + 8NO_3^- + 8H^+ \rightarrow 9H_2O + 5CO_2 + 5HCO_3^- + 4N_2$	-797	-100
$5CH_3CH_2COO^- + 14NO_3^- + 14H^+ \rightarrow 17H_2O + 10CO_2 + 5HCO_3^- + 7N_2$	-1398	-100
$5C_6H_{12}O_6 + 24NO_3^- + 24H^+ \rightarrow 12N_2 + 30CO_2 + 42H_2O$	-2720	-113
DNRA		
$4H_2 + 2NO_3^- + 4H^+ \rightarrow 6H_2O + 2NH_4^+$	-150	-75
$CH_3COO^- + NO_3^- + 2H^+ \rightarrow CO_2 + HCO_3^- + NH_4^+$	-500	-63
$8CH_3CH_2COO^- + 14NO_3^- + 28H^+ \rightarrow 2H_2O + 16CO_2 + 8HCO_3^- + 14NH_4^+$	-878	-63
$8C_6H_{12}O_6 + 24NO_3^- + 48H^+ \rightarrow 12NH_4^+ + 48CO_2 + 12H_2O$	-1835	-77

The intermediates of denitrification are known to inhibit various bacterial species (Klüber and Conrad, 1998b). Nitrogen oxide inhibition of methanogenesis has been reported as follows: $NO>NO_2^->N_2O>NO_3^-$ (Table 2.5). In addition to being the most inhibitory nitrogen oxide, the inhibitory effects of NO are found to be irreversible. On the other hand, inhibition caused by N_2O was partially reversible (Klüber and Conrad, 1998b). It was also found that addition of nitrate strongly affected not only the activity, but also the composition of the methanogenic archaeal community. Nitrate addition resulted in a pronounced suppression of methanogenesis (Scheid et al., 2003).

The inhibitory effect of nitrate on methanogenesis is complicated, because nitrate is further reduced to nitrite, NO, and N_2O, and all these N-oxides can also exert inhibition. It appears that NO is the strongest inhibitor, being effective even at 1 mM, whereas nitrate seems to influence methanogenesis only at relatively high concentrations (e.g., above 20 mM) (Klüber and Conrad, 1998). It is known that some methanogenic species can grow at a nitrate concentration as high as 45 mM (Percheron et al., 1999). Klüber and Conrad (1998) found that the inhibition caused by NO was irreversible, because removal of NO from the culture headspace did not result in resumption of methanogenesis. On the other hand, after N_2O removal form the headspace, methanogenesis partially recovered. It was also found that addition of nitrate strongly affected not only the activity, but also the composition of the methanogenic archaeal

Table 2.5. Inhibitory effects of N-oxides on *Methanosarcina barkeri* and *Methanobacterium bryantii*

N-oxide	Methanogenic Species	N-oxide Concentration	Residual Methanogenic Activity (%)
Nitrate	*Ms. barkeri*	30 mM	24[a]
	Mb. bryantii	30 mM	41[b]
	M. mazei	14.3 mM	54[a]
Nitrite	*Ms. barkeri*	0.1 mM	Complete inhibition[a]
	Mb. bryantii	1 mM	50[a]
Nitric oxide	*Ms. barkeri*	1.7 μM	Complete inhibition[a]
	Mb. bryantii	0.8-1.7 μM	Complete inhibition[a]
Nitrous oxide	*Ms. barkeri*	0.95 mM	10[a]
	Mb. bryantii	> 95 μM	Complete inhibition[a]

[a] Klüber and Conrad, 1998

[b] Clarens et al., 1998

community. The toxicity of NO is a consequence of its reactivity with transition metal proteins and oxygen and its ability to form adducts with amines and thiols of varying stability (Zumft, 1997). Nitrate addition resulted in a pronounced suppression of methanogenesis in rice root incubations (Scheid et al., 2003). It has been found that in the presence of nitrate *Moorella thermoacetica* lacked a membranous b-type cytochrome, which was present in cells grown in the absence of nitrate (Seifritz et al., 2002). The methanogenic activity in a co-immobilized mixed culture system obtained from sewage treatment plant was completely suppressed as long as nitrate or nitrite was present in the system at or above 7.14 and 2.85 mM, respectively (Lin and Chen, 1995).

2.3. Kinetics and Modeling of Anaerobic Processes and Nitrate Reduction

Nitrite reduction is the slowest step of the denitrification process (Zumft, 1997). Nitrate, nitrous oxide, and nitric oxide reduction rates are typically an order of magnitude higher than the nitrite reduction rate. As a result, nitrite accumulation is commonly observed in denitrifying systems. The $K_{c,NOX}$ (i.e., the half-velocity constant for nitrogen oxides) values vary from microorganism to microorganism (Betlach and Tiedje, 1981) and are generally in the range of 3×10^{-8} to 4×10^{-3} kg N/m^3 although a value as high as 0.018 kg N/m^3 has been reported (Knowles, 1982 and Zumft, 1997).

Substrate competition among nitrate reducers and fermenters/methanogens depends on their relative substrate utilization rates and $K_{c,subs}$ (i.e., half-velocity constant for substrate utilization) values. As it has been already mentioned before, denitrifiers have the ability to utilize fermentative/methanogenic substrates such as glucose, glutamic acid, VFAs, and hydrogen. However, the reported utilization rate of glucose and glutamic

acid by denitrifiers is approximately ten fold lower than that for fermentative microorganisms. Although the $K_{c,subs}$ values for denitrifiers are lower than those for the fermentative microorganisms, the denitrifiers may not be able to compete with fermenters for glucose or glutamic acid due to higher utilization rates of these substrates by fermetenters compared to that of the denitrifiers. However, denitrifiers can compete with the methanogens for acetate and hydrogen.

A generalized anaerobic digestion model was developed by the International Water Association (IWA) Anaerobic Digestion Modeling Task Group (Batstone et al., 2002). The IWA Anaerobic Digestion Model No.1 (ADM1) is a structured model, which includes multiple steps describing biochemical and physicochemical processes typically encountered in anaerobic biodegradation. The biochemical processes include extracellular disintegration and hydrolysis and intracellular (biological) fermentation, acidogenesis, acetogenesis, and methanogenesis steps. The disintegration is a first order, non-biological process, which converts composite, polymeric particulate substrate(s) to less complex ("soluble") carbohydrates, proteins, and lipids as well as inert material (Figure 2.1). The complex particulate substrate pool is also used as a pre-lysis repository of the decayed biomass. Carbohydrates, proteins, and lipids are then converted to monomers (monosaccharides, amino acids, and lipids) as a result of complex enzymatic hydrolysis steps, which are also described by first order kinetics. The intracellular steps account for the utilization of monomers, C_{4+} VFA (butyrate and valerate), propionate, acetate, and hydrogen as substrates. Seven separate biological (i.e., microbial) groups are used for the utilization of these substrates. Three inhibition functions defined in the model include pH, hydrogen and free ammonia. The pH inhibition is assumed to be

effective on all biological groups and implemented as one of two alternative empirical equations. Hydrogen and free ammonia inhibitions are assumed to be effective on acetogenic groups and acetoclastic methanogenesis, respectively, and are described by non-competitive inhibition functions. Competitive uptake of butyrate and valerate by a single group of microorganisms is described by an uptake-regulating function. Uptake regulating functions are also used for inorganic nitrogen in order to prevent growth when nitrogen is limited. The physicochemical processes include ion association and dissociation, and gas-liquid exchanges. Methane, carbon dioxide, and hydrogen are assumed to be the three main gas components in the ADM1 (Batstone et al., 2002).

Simple models for the simultaneous methanogenesis and denitrification have been previously developed (Chaudhry and Beg, 1997; Garibay-Orijel et al., 2006). The model developed by Chaudhry and Beg (1997) simulated the simultaneous nitrate reduction and methanogenesis in an upflow sludge bed reactor. The model was simplified by the following assumptions: (a) homogenous biofilm in the reactor; (b) substrate and nitrate are considered to be growth-limiting; (c) a double-substrate Michaelis-Menten type equation was used for denitrification and a single-substrate Michaelis-Menten type equation was used for methanogenesis; (d) transport of chemicals within the biofilm was described by Fick's second law; and (e) interactions of charged molecules with the biofilm were neglected. The model predictions agreed well with the experimental data and the results showed that operation of continuous-flow upflow sludge bed reactors was stable under simultaneous nitrate reduction and methanogenesis conditions. The model developed by Garibay-Orijel et al. (2006) described the amount of electron donor that will be channeled away from methanogenesis to denitrification. In addition, the model

considered the ammonia nitrogen requirements of a system that carries both denitrification and methanogenesis. The model was simplified by the following assumptions: (a) influent COD is completely degradable; (b) nitrate reduction occurs solely by denitrification (i.e., assimilatory or dissimilatory nitrate reduction to ammonia are neglected); (c) the source of nitrogen is ammonia; and (d) nitrification is nonexistent, (i.e., nitrate is not produced from ammonia). The model assumes that denitrifiers use the available carbon and any remaining carbon, after complete nitrate reduction, will be used by methanogens. The model does not consider substrate competition or the possible inhibition of methanogens by N-oxides.

2.4. Problem Identification

Nitrate is released by various industrial and agricultural activities, such as cellophane, pectin, and explosives production, metal finishing, and fertilizer applications. Nitrate is a pollutant in groundwater and surface water, causing a major problem for the supply of drinking water worldwide. Nitrate and/or reduced N-oxides, such as nitrite, nitric oxide, and nitrous oxide, suppress methane production. In recent years, significant research has been conducted on the effect of nitrate reduction on methanogenesis. Most of these studies however were conducted either with pure cultures or with soil microcosms. Research involving mixed methanogenic cultures has only investigated the effect of nitrate. Studies involving concentration-dependent effects of all N-oxides on mixed methanogenic cultures are needed in order to systematically assess the short-term effect of N-oxides on the anaerobic digestion process. Although the effect of sulfide on nitrate reduction has been studied before, research considering the combined effects of

sulfide and nitrate on the inhibition of methanogenesis is lacking. In addition, a systematic assessment of the preferential utilization of different electron donors and the kinetics of nitrate reduction in mixed, overall methanogenic systems is very limited. Therefore, a need exists to understand the effect of nitrate, sulfide, and type of electron donor on process interactions among fermentation, methanogenesis and nitrate reduction on a mixed, methanogenic system.

2.5. Research Objectives

On the basis of the literature information discussed above, research related to the effect of nitrate reduction on methanogenesis is broad but limited. Therefore, a research plan was developed to assess the effect of N-oxides and sulfide on a mixed methanogenic culture, along with the effect of the type of electron donor on the kinetics and pathway of nitrate reduction.

The specific objectives of this research were:

1) Investigation the potential inhibitory effects of the type and concentration of N-oxides on methanogenesis, anaerobic fermentative processes, and nitrate reduction.
2) Assessment of the effect of different carbon/electron donor sources on nitrate reduction and methanogenesis.
3) Assessment of the effect of sulfide on nitrate reduction in a mixed methanogenic culture.
4) Evaluation of the kinetics and modeling of the simultaneous anaerobic fermentation and nitrate reduction in an overall methanogenic system.

CHAPTER 3

ANALYTICAL METHODS AND GENERAL PROCEDURES

3.1. Analytical Methods

3.1.1. pH

Measurement of pH was performed using the potentiometric method with a digital pH/milivolt meter (Orion Digital pH/milivolt Meter, Model 611) and a gel-filled combination pH electrode (Fisher Scientific, Pittsburgh, PA). The meter was calibrated prior to each use using standard buffer solutions of pH 4, 7, and 10 (Fisher Scientific). Before and between sample readings, the electrode was rinsed with deionized water (DI) and gently dried with Kimpex wipes (Fisher Scientific). The electrode was stored in a pH electrode storage solution (Fisher Scientific) of pH 4 when not in use. Although the sensitivity of the meter display was 0.01 units, measurements were recorded to only 0.1 pH units.

3.1.2. Oxidation-Reduction Potential (ORP)

Oxidation-reduction potential measurements were performed using an Orion Digital pH/milivolt Meter, Model 611 and a Sensorex combination ORP electrode (platinum electrode with a Ag/AgCl reference electrode in a 3.5 M KCl gel). The meter and electrode output were periodically checked using an ORP reference solution [0.10 M ferrous ammonia sulfate, 0.10 M ferric ammonium sulfate, and 1.0 M sulfuric acid (Light

solution; APHA, 1998)]. The difference between the meter ORP reading and the theoretical value of the reference solution (455 mV at 25°C) was taken into account in all sample ORP measurements. To obtain ORP values with reference to the standard hydrogen electrode (SHE), a correction factor of +220 mV was added to the instrument values. ORP measurements were conducted by taking a sample by syringe and transferring it into a 5-mL serum bottle in which the electrode fitted tightly. Then, the electrode was quickly inserted into the serum bottle. During the ORP measurement extreme care was taken in order to prevent the intrusion of air bubbles to the serum bottle and, thus to minimize atmospheric oxidation. The ORP measurement was recorded when the instrument reading was not changing more than 1 mV/min.

3.1.3. Total and Volatile Solids

Total solids content of samples were determined according to procedures outlined in *Standard Methods* (APHA, 1998). Samples were weighed in pre-ignited (550ºC) and cooled ceramic crucibles using an Ohaus AP250D Analytical Balance (precise to ± 0.02 mg up to 52 g, and to ± 0.1 mg between 52 and 210 g). The samples were then dried at 105ºC in a Fisher Isotemp Model 750G oven. After drying, the crucibles were transferred to a desiccator until cooled, and then the dry weight was measured. If total volatile solids were to be determined, the crucibles were transferred to a Fisher Isotemp Model 550-126 muffle furnace and ignited at 550ºC. After ignition, the samples were cooled in a desiccator and the residual solids weight was measured.

3.1.4. Ammonia

The titrimetric method described in *Standard Methods* (APHA, 1998) was followed for ammonia determination. Distillation was conducted in a Labconco Rapid Distillation Unit. A 10 mL sample, 0.5 mL borate buffer solution, and 0.3 mL 6 N NaOH solutions were added simultaneously to the distillation unit and then about 45 mL distillate was collected in a beaker containing 10 mL indicating boric acid solution. Collected distillate was then diluted up to 125 mL with DI water and the ammonia concentration was determined by titrating the distillate with 0.02 N H_2SO_4 titrant. Aliquots of 10 mL DI water were used as blank samples. Ammonia recoveries were determined with a standard NH_4Cl solution and varied from 94 to 99 %.

3.1.5. Gas Chromatography

3.1.5.1. Thermal Conductivity Detection

The gas produced by anaerobic cultures was monitored for methane (CH_4), carbon dioxide (CO_2), nitric oxide (NO), nitrous oxide (N_2O), nitrogen (N_2), and hydrogen sulfide (H_2S) concentrations using a GC unit (Agilent Technologies, Model 6890N; Agilent Technologies, Inc., Palo Alto, CA) equipped with two columns and two thermal conductivity detectors. Methane, NO, and N_2 were separated with a 15-m HP-Molesieve fused silica, 0.53 mm i.d., and 50-µm film thickness column (Agilent Technologies, Inc.). Separation of CO_2, N_2O, and H_2S was performed with a 25-m CP-PoraPLOT Q fused silica, 0.53 mm i.d., and 20-µm film thickness column (Varian, Inc., Palo Alto, CA). Helium was used as the carrier gas at a constant flow rate of 6 mL/min. The 10:1 split injector was maintained at 150^{o}C, the oven was set at 40^{o}C and the detector temperature

was set at 150ºC. The minimum gas-phase detection limit for CH_4, CO_2, NO, N_2O, N_2, and H_2S was 0.5, 0.8, 0.5, 0.007, 0.05 and 0.1 mL/L, respectively.

3.1.5.2. Flame Ionization Detection

The samples were analyzed for VFAs with 1 µL direct liquid injection using an HP 5890 Series II gas chromatograph (Hewlett Packard, Palo Alto, CA) equipped with a flame ionization detector and a 30-m Stabilwax-DA column with 0.53-mm i.d. and 0.25-µm film thickness (Restek Company, Bellefonte, PA, USA). The injector and detector temperatures were set at 300ºC. The temperature program used was as follows: 90ºC for 0.5 min, increased by 2ºC /min to 100ºC, 6ºC /min to 130ºC, 15ºC/min to 230ºC, and held at 230ºC for 7 min. The total run time was 30 min. Nitrogen was used as a carrier gas at a constant flow rate of 4.8 mL/min. The minimum detection limits for the VFAs were as follows (mg/L): acetic, 7; propionic, 4; isobutyric and butyric, 3; and isovaleric and valeric, 2.5.

Liquid samples were centrifuged and filtered through 0.2 µm syringe filters for VFA measurements. Samples were then diluted with a 2.5% (v/v) H_3PO_4 solution (sample:acid, 2:1 v/v) and stored in 1.5-mL glass autosampler vials at 4ºC until analysis. Calibration curves were prepared by using pure standards dissolved in DI water containing 0.83% phosphoric acid.

3.1.6. Total Gas Production

Total gas production during incubation of large volume cultures (2 to 9-L culture reactors) was measured by periodically releasing the accumulated gas into a graduated

burette manometer containing an acidified brine solution (10% NaCl w/v and 2% H_2SO_4 v/v). By the use of this manometer, the culture headspace pressure was equilibrated to atmospheric pressure at the culture incubation temperature, and the total gas volume was determined. Total gas production in small cultures (vials, tubes, or serum bottles) was measured using a digital pressure gauge (ColeParmer, IL, USA) with 2% accuracy.

3.1.7. Total and Soluble Sulfide

Total and soluble sulfide measurements in samples were determined using an acid-volatile sulfur measurement procedure similar to that described by Bagley and Gossett (1990). Prior to both total and soluble sulfide measurements, 1 mL 6 N H_2SO_4 solution was added to 12-mL glass vials, capped with Teflon-lined septa and aluminum crimps, and flushed with Helium gas for 2 minutes. Total sulfide analysis was conducted by injecting a 5 mL sample into each vial. The bottles were vigorously shaken for several minutes and then incubated in an inverted position at 35ºC for one hour. After incubation, the headspace over each sample was analyzed for hydrogen sulfide by injecting 100 µL samples of headspace gas into the GC (see section 3.1.5.1, above).

Samples for soluble sulfide measurements were anaerobically centrifuged and filtered thorough 0.2 µm Whatman syringe filters (Fisher Scientific). Extreme care was taken to prevent possible oxidation of sulfide by oxygen, which may be introduced via air. Filtered samples were injected to pre-flushed vials containing 1 mL 6 N H_2SO_4, vigorously shaken and incubated at 35^{o}C for one hour. Soluble sulfide was measured using the same procedure described for the total sulfide measurements (i.e., gas-phase H_2S analysis by GC/TCD).

The hydrogen sulfide peak areas were calibrated against total sulfide mass using standards containing sodium sulfide dissolved in DI water. Each calibration sample was created by injecting 5 mL of sulfide solution into a 1 mL 6 N H_2SO_4 containing, helium-flushed serum bottle capped with a Teflon-lined septum and an aluminum crimp. The vials were vigorously shaken and incubated for one hour at 35^oC, and analyzed using the same procedure described for the sample measurements. Due to the rapid oxidation of sulfide, right after acidifying each standard sample, a second aliquot of the standard was titrated using the iodometric method described in the *Standard Methods* (APHA, 1998) to determine the exact sulfide concentration at the time of analysis. A total sulfide curve was created by plotting sulfide peak area (TCD output) of the headspace sample for each acidified standard bottle against the mass of sulfide added to it. A sample calibration curve for this analysis is shown in Figure 3.1.

3.1.8. Ion Chromatography

Nitrate (NO_3^-), nitrite (NO_2^-), and sulfate (SO_4^{2-}) concentrations were determined using a Dionex DX-100 ion chromatography unit (Dionex Coorporation, Sunnyvale, CA, USA) equipped with a conductivity detector, a Dionex IonPac AG14A (4x50 mm) precolumn, and a Dionex IonPac AS14A (4x250 mm) analytical column. The eluent was a mixture of 8 mM Na_2CO_3 and 1mM $NaHCO_3$ used at an isocratic flow rate of 1mL/min. Autosuppression mode was used. Calibration curves were created using standards prepared by dissolving reagent grade sodium salts of each analyte in DI water. All standards and samples were filtered through 0.2 µm membrane filters prior to

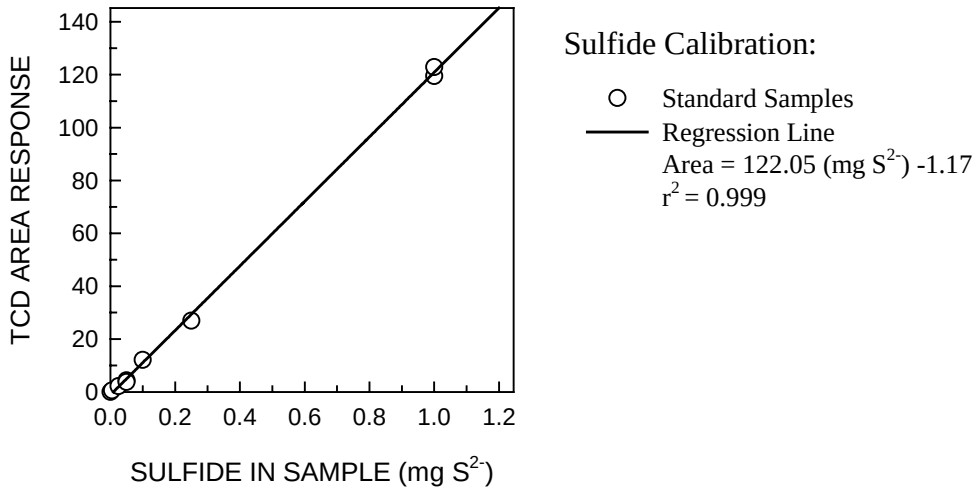

Figure 3.1. Sample calibration curve for sulfide measurement (via gas-phase H_2S).

injection. The injection volume was 1 mL. The minimum detection limits for NO_3^-, NO_2^-, and SO_4^{2-} were 0.05, 0.1 mg N/L, and 0.07 mg S/L, respectively.

3.2. General Procedures

3.2.1. Culture Media

3.2.1.1. Sulfide-Free Media

Anaerobic cultures were developed and sustained in growth media which supplied all necessary nutrients, trace metals, and vitamins. The composition of culture media is shown in Tables 3.1. Culture media were prepared by adding the first seven ingredients in Table 3.1 to 8 L DI water in 9-L Pyrex serum bottles. The media bottles were covered with aluminum foil and steam autoclaved (121°C; 45 min). Stoppers fitted with Nalgene rigid tubing for headspace gas exchange and dispensing the media were also autoclaved wrapped in aluminum foil alongside the media bottles.

After autoclaving, the stoppers were clammed in place on top of the media bottles, which were then flushed with helium (through the Nalgene tube reaching the bottom of the bottle – later to be used for dispensing media) for at least one hour while being mixed using magnetic stir plates. This step was performed to strip most of the dissolved oxygen from the media as it cooled. Vitamins and sodium bicarbonate were added and the media were thoroughly mixed until they reached room temperature. The pH was checked to confirm that it was in the range of 7.2 to 7.6. The media bottles were covered with black plastic bags in order to protect light-labile media components.

3.2.1.2. Sulfide-Amended Media

The first seven components listed in Table 3.1 were added to 8 L DI water in 9-L Pyrex bottles. In addition, resazurin was added as a redox indicator, which is colorless when reduced and bright pink when oxidized (at neutral pH; at low pH values it is blue when oxidized). Resazurin was used in the media as a visual aid so that it could be quickly confirmed when the media were anaerobic. Previously described autoclaving and flushing procedures were applied during the preparation of these media. However, after flushing, and while the media were still warm, sodium sulfide (0.5 g/L $Na_2S \cdot 9H_2O$) was added as a reducing agent. Vitamins and sodium bicarbonate was added after the media had reached room temperature. The pH was checked to confirm that it was in the range of 7.2 to 7.6. The media bottles were covered with black plastic bags in order to protect light-labile media components.

Table 3.1. Media composition of mixed fermentative/methanogenic cultures

Compound	**Concentration**
K_2HPO_4	0.9 g/L
KH_2PO_4	0.5 g/L
NH_4Cl	0.5 g/L
$CaCl_2 \cdot 2H_2O$	0.1 g/L
$MgCl_2 \cdot 6H_2O$	0.2 g/L
$FeCl_2 \cdot 4H_2O$	0.1 g/L
Trace metal stock solution	1 mL/L
Resazurin Stock[a]	2 mL/L
Vitamin stock solution	1 mL/L
$NaHCO_3$	3.5 g/L
$Na_2S \cdot 9H_2O$[a]	0.5 g/L
Trace metal stock solution	**Concentration (g/L)**
$ZnCl_2$	0.5
$MnCl_2 \cdot 4H_2O$	0.3
H_3BO_3	3
$CoCl_2 \cdot 6H_2O$	2
$CuCl_2 \cdot 2H_2O$	0.1
$NiSO_4 \cdot 6H_2O$	0.2
$Na_2MoO_4 \cdot 2H_2O$	0.3
Vitamin stock solution	**Concentration (g/L)**
Biotin	0.2
Folic Acid	0.2
Pyridoxine Hydrochloride	1
Riboflavin	0.5
Thiamine	0.5
Nicotinic Acid	0.5
Pantothenic Acid	0.5
Vitamin B_{12}	0.01
p-Aminobenzoic Acid	0.5
Thioctic Acid	0.5
Resazurin stock solution	1 g/L

[a] Added only to the sulfide-amended media.

CHAPTER 4

INHIBITORY EFFECTS OF NITROGEN OXIDES ON TWO MIXED METHANOGENIC CULTURES

4.1. Introduction

In recent years, significant research has been conducted on the effect of nitrate reduction on methanogenesis. Most of these studies have been conducted either with pure cultures (Klüber and Conrad, 1998a; Clarens et al., 1998) or with soil microcosms (Klüber and Conrad, 1998b; van Bodegom and Stams, 1999; Roy and Conrad, 1999; Chidthaisong and Conrad, 2000). Research involving mixed methanogenic cultures has only investigated the effect of nitrate (Akunna et al., 1994; Hendriksen and Ahring, 1996). Studies involving concentration-dependent effects of all N-oxides on mixed methanogenic cultures are needed in order to systematically assess the short-term effect of N-oxides on the anaerobic digestion process. In addition, possible long-term effects of nitrate on methanogenesis are not sufficiently explored in sulfide-free or sulfide-acclimated cultures are not sufficiently explored.

The objectives of the research reported here were to: (a) assess the effect of nitrate, nitrite, nitric oxide, and nitrous oxide on a mixed, mesophilic (35^{o}C) methanogenic culture as a function of N-oxide concentration; (b) investigate the long-term effect of repetitive nitrate addition to the mixed methanogenic culture; and (c)

investigate the long-term effect of repetitive nitrate additions to a sulfide-acclimated, mixed methanogenic culture.

4.2. Materials and Methods

4.2.1. Sulfide-Free and Sulfide-Acclimated Enriched Methanogenic Cultures

Assays were conducted using two mixed, methanogenic cultures: a *sulfide-free culture* enriched in sulfide-free media, and a *sulfide-acclimated culture* enriched in sulfide-bearing media. Steady-state pH values of the sulfide-free and sulfide-acclimated cultures were 6.9 ± 0.1 and 7.1 ± 0.1 (mean ± standard deviation), respectively. Both cultures were developed with an inoculum obtained from a mesophilic (35^{o}C), municipal anaerobic digester, and were fed with dextrin/peptone (4 g/L dextrin, 2 g/L peptone in the feed solution) and nutrient media (see Chapter 3). The peptone used (Sigma-Aldrich) contains 8% nitrogen and degradation of peptone may yield a maximum of 32 mg NH_4^+-N/L in every culture feeding. In addition, ammonia contribution of the media is 130.2 mg NH_4^+-N/L. Therefore, the total amount of ammonia supplied by the feed and media was 194 mg NH_4^+-N/L when the culture was fed twice a week. The total sulfide concentration provided by the trace metals and vitamins in the media was 0.2 mg S/L. Therefore, the total amount of sulfide in the sulfide-free and sulfide-amended media was 0.2 and 67 mg S/L, respectively. The two methanogenic cultures (6 L liquid volume each) were maintained at 35ºC and were fed twice a week with a hydraulic (and solids) retention time of 35 days and were continuously mixed using a magnetic stirrer. Both cultures were maintained under the above-stated conditions for over two years and three years before the initiation of this study, respectively. The steady-state gas-phase CH_4 and CO_2 and the

total solids (TS) and volatile solids (VS) concentrations of the sulfide-free culture were 59.7± 0.8%, 40.3 ± 0.9%, 5,524 ± 426 mg/L, and 1,810 ± 84 mg/L (mean ± standard deviation), respectively. Likewise, the steady-state gas-phase CH_4 and CO_2 and the TS and VS concentrations of the sulfide-acclimated culture were 60.7 ± 0.8%, 39.2 ± 0.4%, 6,900 ± 300 mg/L, and 2,200 ± 100 mg/L (mean ± standard deviation), respectively.

4.2.2. Abiotic Nitrate Reduction

In order to assess the possibility of abiotic nitrate reduction by any media components, an abiotic control assay was conducted using 160-mL serum bottles (100 mL liquid volume). Serum bottles were sealed with rubber stoppers and aluminum crimps and pre-flushed with helium gas. Three types of autoclaved controls were used: deionized water (DI), sulfide-free culture media, and sulfide-free culture. All cultures were autoclaved at 121^{o}C for 1 hour twice in two consecutive days. Autoclaved cultures were amended with 40 mg N/L nitrate. Both the sulfide-free media and culture were then monitored for their ability to reduce nitrate. Incubation of the controls was carried out in the dark at 35ºC under continuous mixing using magnetic mixers.

4.2.3. Short-Term Inhibition Assays with a Sulfide-Free Culture

Batch assays to test the effect of nitrate and nitrite on methanogenesis were performed using 160-mL serum bottles (112 mL liquid volume) sealed with rubber stoppers and aluminum crimps and pre-flushed with helium gas. An aliquot of 70 mL mixed, sulfide-free methanogenic culture and culture media were anaerobically transferred to each serum bottle, and a dextrin/peptone mixture was added resulting in an

initial concentration of 946/473 mg/L (1,009/493 mg COD/L), respectively. Replicate sacrificial cultures were setup for the explicit measurement of initial pH and biomass concentration. The initial biomass concentration in the serum bottles was 1,120 ± 40 mg VS/L and 1,022 ± 17 mg VS/L for the nitrate- and nitrite-amended cultures, respectively. Aliquots of $NaNO_3$ or $NaNO_2$ stock solutions were added to each serum bottle resulting in initial concentrations ranging from 10 to 350 mg N/L nitrate and 17 to 500 mg N/L nitrite. A possible sodium effect as a result of $NaNO_3$ and $NaNO_2$ addition was tested in the long-term inhibition assay (see Section 4.2.4 below) with no adverse effect observed. Therefore, the effect of sodium was not tested in the short-term inhibition assays. The initial concentrations of nitrate and nitrite were selected to arrive at the same electron equivalents (i.e., COD) required for the complete N-oxide reduction to N_2. The COD required for the complete N-oxide reduction (COD_{nox}) was calculated based on 2.857 mg COD/mg NO_3^--N and 1.714 mg COD/mg NO_2^--N (Table 4.1). The COD requirement for the growth of denitrifiers (COD_{xnox}) was also calculated using f_e and f_s values based on thermodynamic and bioenergetic principles (Rittmann and McCarty, 2001), where f_e and f_s are the fractions of the electron donor (electron equivalent basis) used for energy generation (i.e., N-oxide reduction) and microbial growth, respectively. The f_e and f_s Values were calculated based on single step N-oxide reduction to N_2 assuming volatile fatty acids (VFAs) as the electron donors. Calculated f_e/f_s values for nitrate and nitrite were 0.37/0.63 and 0.34/0.66, respectively, neglecting microbial decay. Although the COD_{nox} values for the 350 mg N/L nitrate- and 500 mg N/L nitrite-amended cultures are less than the total initial COD supplied (COD_{in}; 1,503 mg/L), when growth of denitrifiers is accounted for, the total COD required for the denitrification process (COD_{total}) exceeds

Table 4.1. COD requirements for N-oxide reduction and growth of denitrifiers for all N-oxide amended cultures used in this study.

Culture series	COD required (mg/L)		
	N-oxide reduction (COD_{nox})[a]	Growth of denitrifiers (COD_{xnox})[b]	Total (COD_{total})
Nitrate (mg N/L)			
10	29	48	77
30	86	145	231
75	214	365	579
150	429	729	1158
350	1000	1703	2703
Nitrite (mg N/L)			
17	29	57	86
50	86	166	252
125	214	416	630
250	429	831	1260
500	857	1664	2521
Nitric oxide (mg N/L)			
0.02	0.5	2.2	2.7
0.16	4.1	17.9	22
0.80	21	88	109
Nitrous oxide (mg N/L)			
19	27	70	97
48	64	163	227
96	115	297	412
191	195	502	697

[a] Calculated based on 2.857 mg COD/ mg NO_3^--N , 1.714 mg COD/ mg NO_2^--N, 1.143 mg COD/ mg NO-N and, 0.571 mg COD/ mg N_2O-N.
[b] Calculated based on theoretical f_e and f_s values (see text).

the COD_{in} (Table 4.1). Under these conditions, incomplete N-oxide reduction is expected to occur due to limitation of the carbon/electron donor source in the 350 mg N/L nitrate- and 500 mg N/L nitrite-amended cultures. However, it should be noted that the f_e and f_s calculations were carried out with the assumption that microbial decay does not take place. Nevertheless, cells decay and a fraction of the electrons used for microbial growth are transferred to the electron acceptor to generate more energy. Therefore, although the theoretically required COD_{total} is higher than the COD_{in}, complete N-oxide reduction can take place due to the additional electron source made available by the decay of biomass.

Batch assays to investigate the effect of NO and N_2O were carried out in sacrificial 26-mL glass serum tubes (14 mL liquid volume). An aliquot of 9.2 mL mixed, methanogenic culture and culture media were added to each tube and a dextrin/peptone mixture was added resulting in an initial concentration of 1,044/522 mg/L (1,114/544 mg COD/L), respectively. Replicate sacrificial tubes were setup for the explicit measurement of initial pH and biomass concentration. The initial biomass concentration in the tubes was 1,078 ± 14 mg VS/L and 1,100 ± 12 mg VS/L for the NO- and N_2O-amended cultures, respectively. Pure NO and N_2O gases were added to result in initial aqueous concentrations ranging from 0.02 to 0.8 mg N/L NO and 19 to 191 mg N/L N_2O, respectively. The aqueous NO and N_2O concentrations were calculated using the Henry's law constants of 1.838×10^{-3} and 2.49×10^{-2} M/atm (at 35°C) for NO and N_2O, respectively (Stumm and Morgan, 1996). Hereafter, NO and N_2O concentrations in the cultures refer to aqueous (i.e., dissolved) concentrations. Low initial NO concentrations were used because of its known inhibitory effect on methanogens (Klüber and Conrad, 1998a). COD calculations were performed using the same approach as that described

above for the nitrate and nitrite amended cultures. The COD required for the complete NO and N_2O reduction to N_2 is 1.143 mg COD/mg NO-N and 0.571 mg COD/mg N_2O-N, respectively. The calculated f_e/f_s values were 0.19/0.81 and 0.28/0.72 for nitric oxide and nitrous oxide reduction, respectively. As shown in Table 4.1, COD_{total} values for the nitric oxide-amended cultures were significantly lower than the COD_{in} (1,658 mg/L) due to low initial NO concentrations. The initial concentration of N_2O was selected to be lower than nitrate due to the reported higher inhibitory effect of N_2O compared to that of nitrate (Klüber and Conrad, 1998a). Therefore, for all applied N_2O concentrations, COD_{total} values were lower than the COD_{in} (Table 4.1). In order to differentiate between methane and NO peaks during the gas chromatographic analysis, one culture series prepared with the highest initial NO concentration (0.8 mg N/L) was amended with 50 mM of 2-bromoethanesulfonate (BES, a known inhibitor of methanogens) two days prior to the addition of NO and the initiation of the incubation.

Control cultures for all N-oxide assays were set up with the methanogenic culture and the same organic feed solution, without any N-oxide amendment. Replicates for all culture series were not prepared due to the relatively large number of conditions examined and time required for frequent sampling and analysis. All incubations were carried out in the dark at 35ºC. A preliminary experiment showed that in the absence of mixing, significantly higher accumulation of denitrification intermediates was observed. Therefore, all serum bottles and tubes were mixed continuously using a tumbler at 4 rpm. pH, VFA, and nitrate/nitrite measurements were carried out by removing liquid samples from serum bottles or sacrificing glass tubes for the nitrate-/nitrite- or NO-/N_2O-amended cultures, respectively.

4.2.4. Long-Term Inhibition Assays with a Sulfide-Free Culture

In order to investigate the effect of prolonged exposure of the sulfide-free methanogenic culture to nitrate, two cultures were prepared using 2.3-L glass reactors (1.75 L liquid volume). An aliquot of 1.25 L methanogenic culture was anaerobically transferred to each reactor along with culture media. The initial biomass concentration was 1,549 ± 28 mg VS/L. This assay was conducted in four feeding cycles. Dextrin/peptone mixture was used as the carbon/electron donor source at an initial concentration of 1,028/514 mg/L (1,097/536 mg COD/L) in all four feeding cycles. In the second and fourth feeding cycle, one of the cultures (test culture) was amended with 300 mg nitrate-N/L. In order to account for any possible sodium effect due to $NaNO_3$ addition to the test culture, an aliquot of a NaCl stock solution was added to the control culture (1,254 mg /L final concentration) in the second and fourth feeding cycles, for which the test culture was amended with nitrate. Incubation of these cultures was carried out in the dark at 35ºC with continuous mixing using magnetic stirrers. Before the onset of each feeding cycle, the culture volume was adjusted to 1.75 L with the addition of culture media and the headspace was flushed with helium gas.

4.2.5. Long-Term Inhibition Assays with a Sulfide-Acclimated Culture

In order to investigate the effect of prolonged exposure of the sulfide-acclimated methanogenic culture to nitrate, two assays were conducted. In the first assay, three cultures were prepared using 2.3-L glass reactors (1.85 L liquid volume). An aliquot of 1.25 L methanogenic culture was anaerobically transferred to each reactor along with culture media. The initial biomass concentration was 2,767 ± 21 mg VS/L. This assay

was conducted in six feeding cycles. Dextrin/peptone mixture was used as the carbon/electron donor source at an initial concentration of 1,054/527 mg/L (1,124/562 mg COD/L) in all six feeding cycles to all three cultures and was added weekly. All three cultures were fed with D/P without any nitrate addition in the first feeding cycle in order to prove that the activity of all three cultures was the same. One of the cultures was used as a nitrate-free control culture and the other two were fed with low- and high-levels of nitrate at the beginning of the second through the sixth feeding cycles. The low-level nitrate-amended culture had an initial nitrate concentration of 10 mg N/L. However, the high-level nitrate-amended culture had an initial nitrate concentration of 10 mg N/L in the second and third feeding cycles and the initial nitrate concentration was increased to 25 mg N/L in the fourth, fifth, and sixth feeding cycles. Incubation of these cultures was carried out in the dark at 35ºC with continuous mixing using magnetic stirrers. Before the onset of each feeding cycle, the culture volume was adjusted to 1.85 L with the addition of culture media.

In the second assay, three cultures were prepared and used as control, low-level, and high-level nitrate-amended cultures as described above. This assay was conducted in five feeding cycles. The low- and high-level nitrate-amended cultures were amended with different nitrate concentrations in every cycle. Different from the first assay, the carbon source and nitrate were supplied daily. A feed solution mixture containing D/P and nitrate (53 mL) was added once a day. The D/P concentration per 7-d feeding was 1,067/534 mg/L (1,138/570 mg COD/L, or an equivalent of 7480 mg COD/L in the influent) and was added to all three cultures. The low- and high-level nitrate-amended cultures were amended with 10 mg N/L·week nitrate (50 mg N/L in the influent) and 25 mg N/L·week

nitrate (125 mg N/L in the influent) in every cycle, respectively. Incubation of these cultures was carried out in the dark at 35ºC with continuous mixing using magnetic stirrers.

4.2. Results and Discussion

4.3.1. Assessment of Abiotic Nitrate Reduction

The assay testing the possibility of abiotic nitrate reduction lasted for 20 days. In all the autoclaved controls (DI, sulfide-free media, and sulfide-free culture), a slight decrease in the nitrate concentration occurred at the beginning of the incubation. However, the nitrate concentration remained the same during the incubation period regardless of the presence of sulfide (Figure 4.1). Nitrite was not produced during the incubation period in any of the autoclaved controls. Likewise, gas production was not observed in these controls. Therefore, reduction of nitrate in the sulfide-free, active cultures was biologically mediated.

4.3.2. Short-Term Inhibition Assessment in a Sulfide-Free Culture

The effect of nitrate, nitrite, nitric oxide, and nitrous oxide on the sulfide-free mixed methanogenic culture was assessed as a function of initial concentrations ranging from 10 to 350, 17 to 500, 0.02 to 0.8, and 19 to 191 mg N/L, respectively. The initial pH measurements were conducted by removing liquid sample from the nitrate- and nitrite-amended cultures and sacrificial tubes were used for the NO- and N_2O-amended cultures. The initial pH values were 7.11 ± 0.01, 7.11 ± 0.05, 7.25 ± 0.02, and 7.25 ± 0.01 (mean ±

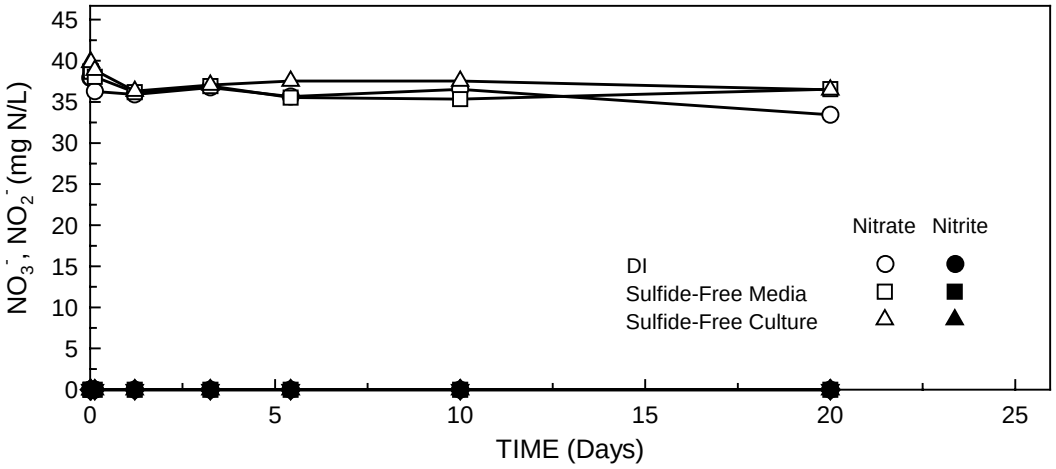

Figure 4.1. Nitrate and nitrite concentrations in autoclaved controls.

standard deviation) in the nitrate, nitrite, nitric oxide, and nitrous oxide amended cultures, respectively. The final pH values were 7.08, 7.25, 7.32, 7.37, 7.42, and 7.56 in the 0, 10, 30, 75, 150, and 350 mg N/L nitrate-amended; 7.07, 7.32, 7.41, 7.49, 7.56, and 8.06 in the 0, 17, 50, 125, 250, and 500 mg N/L nitrite-amended; 7.04, 7.03, 7.04, 6.96 in the 0, 0.02, 0.16, and 0.8 mg N/L NO-amended; and 7.07, 7.09, 7.1, 7.12, and 7.01 in the 0, 19, 48, 96, and 191 mg N/L N_2O-amended cultures. An increase in the pH values as a result of denitrification was observed in the nitrate-, nitrite-, and nitrous oxide-amended cultures as compared to the control cultures (N-oxide free cultures), which became more apparent as the initial N-oxide concentration increased. However, the final pH values in the NO-amended cultures were very close to that of the control culture, which is attributed to the low initial NO concentrations used. Ammonia production was not observed in any of the N-oxide amended cultures at the end of the incubation, indicating that the primary pathway of nitrate reduction was denitrification, and not DNRA. Due to the similarities in the N-oxide reduction profiles, N-oxide reduction data for only the 350 mg N/L nitrate-, 500 mg N/L nitrite-, 0.8 mg N/L NO-, and 191 mg N/L N_2O-amended cultures are reported here. Similarly, VFA production and consumption profiles are only reported for the control and the above mentioned cultures. Methane recovery was monitored and the initial volumetric methane production rates were calculated using linear regression starting at the recovery time. These data are reported in Table 4.2 for all N-oxide amended cultures.

The assay testing the effect of nitrate on methanogenesis lasted for 13 days. Methanogenesis was observed from the beginning of the incubation in the 10 and 30 mg N/L nitrate-amended cultures (Figure 4.2A). Addition of nitrate resulted in an immediate

suppression of methane production in the 75, 150, and 350 mg N/L nitrate-amended cultures (Figure 4.2A). As reported in Table 4.2, recovery of methanogenesis in the 150 and 350 mg N/L nitrate-amended cultures was concomitant to the complete reduction of all N-oxides to N_2 in these cultures (0.9 and 1.2 d, respectively), whereas in the 75 mg N/L nitrate-amended culture, methane production was observed 0.3 d (7.2 h) before the disappearance of N-oxides. Production of methane from the beginning of the incubation in the 10 and 30 mg N/L nitrate-amended cultures along with the recovery of methanogenesis before the complete reduction of N-oxides in the 75 mg N/L nitrate-amended culture suggest that at low concentrations, nitrate is not inhibitory to the methanogenesis. A decrease in the initial methane production rate was observed as the initial nitrate concentration increased (Table 4.2). The observed inhibition of methanogenesis at higher nitrate concentrations is attributed to the increased accumulation of denitrification intermediates. Inhibition of methanogenesis due to reduced denitrification intermediates rather than nitrate has been previously reported (Clarens et al, 1998; Klüber and Conrad, 1998a). The extent of methane production in the nitrate-amended cultures was lower than that of the control culture due to the electron equivalents used for nitrate reduction (Figure 4.2A). According to the calculated theoretical COD requirements for nitrate reduction and growth of denitrifiers, the initial supplied COD was not enough for the complete nitrate reduction in the 350 mg N/L nitrate-amended culture (Table 4.1). However, not only was complete nitrate reduction observed, but also 26.9% of the total COD processed was accounted for as methane in the 350 mg N/L nitrate-amended culture (Figure 4.2B and Table 4.2). The amount of COD converted to methane in the control culture was 152.6 mg. However, 41.1 mg and 112 mg

Table 4.2. Initial methane production rate and COD utilization in sulfide-free mixed methanogenic cultures amended with different N-oxide compounds at different concentrations.

	N-oxide reduction	Methane production				COD Processed (%)			
Culture series	Time (d)[a]	Time (d)[c]	Initial rate (mL/L-d)[d]	r^2	Normalized rate (%)[e]	CH_4	VFAs	N-oxide reduction[f]	Total[g]
Nitrate (mg N/L)									
0	0	0	182 ± 21	0.948	100	100.0	0	0	100.0
10	0.6	0	179 ± 13	0.984	98	100.2	0	2.1	102.3
30	1.2	0	166 ± 17	0.989	91	90.3	0	6.3	96.6
75	1.2	0.9	157 ± 7	0.998	86	76.1	0	15.7	91.8
150	1.2	1.2	95 ± 0	1.000	52	66.4	0	31.5	97.9
350	4.3	4.3	34 ± 13	0.874	18	26.9	0	73.5	100.4
Nitrite (mg N/L)									
0	0	0	136 ± 5.4	0.994	100	100.0	0	0	100.0
17	0.1	0.1	102 ± 7	0.978	75	94.4	0	1.9	96.3
50	0.8	0.8	27 ± 2	0.983	20	89.4	0	5.4	94.8
125	1.6	1.6	26 ± 7	0.935	19	77.8	0	13.6	91.3
250	2.8	10	18 ± 1	0.998	14	60.2	0	27.2	87.3
500	10.0	18	2.6 ± 0.9	0.949	2	14.0	20.7	54.3	89.0
Nitric oxide (mg N/L)									
0	0	0	136 ± 21	0.899	100	100.0	0	0	100.0
0.02	ND[b]	0	143 ± 14	0.968	105	91.9	0	0.03	91.9
0.16	ND	0	64 ± 14	0.877	47	88.4	0	0.2	88.6
0.80	16.5	ND	0		0	0	69	1.2	70.2
Nitrous oxide (mg N/L)									
0	0	0	207 ± 28	0.905	100	100.0	0	0	100.0
19	1.2	0.17	100 ± 7	0.942	48	98.8	0	0.7	99.5
48	1.2	0.9	107 ± 14	0.923	53	95.2	0	2.1	97.3
96	1.2	1.17	121 ± 7	0.998	58	96.4	0	3.1	99.5
191	2.0	1.2	93 ± 7	0.988	45	96.4	0	4.1	100.5

[a] Time required for the complete N-oxide reduction to N_2.
[b] ND, not detected.
[c] Incubation time at which methane was first detected.
[d] Results of linear regression (mean ± standard deviation; $n \geq 3$) of single culture data starting at the recovery time.
[e] Normalized to the initial methane production rate of the control culture observed at each assay.
[f] Fraction of COD utilized for the complete utilization of each N-oxide to N_2 neglecting microbial growth (calculated).
[g] Normalized to the total COD utilized for methane production in the control culture.

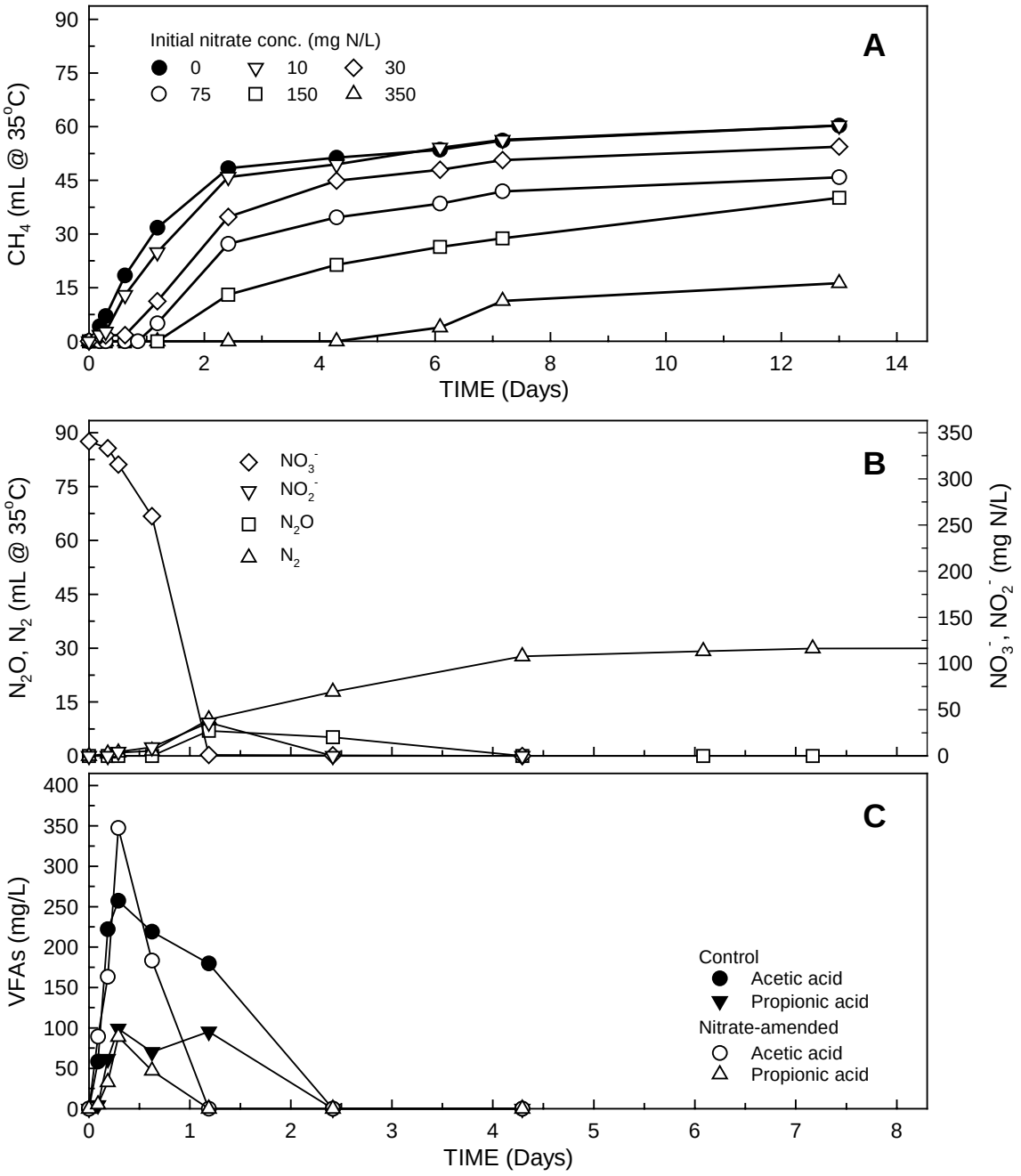

Figure 4.2. Effect of nitrate on the sulfide-free mixed methanogenic culture. (A) Cumulative methane production at different initial nitrate concentrations, (B) production and consumption profiles of N-oxide species in the 350 mg N/L nitrate-amended culture, (C) VFA production and consumption profiles in the control and 350 mg N/L nitrate-amended cultures.

of the initial COD was used for methane production and the complete conversion of nitrate to N_2 in the 350 mg N/L nitrate-amended culture, respectively, resulting in total COD consumption of 153.1 mg. These data suggest that because of the prolonged incubation time, biomass decay and fermentation provided needed electron equivalents for the complete reduction of nitrate and the production of methane. In all nitrate-amended cultures, the total processed COD was very close to that of the control culture, indicating that all initially supplied COD was used for N-oxide reduction and methane production (Table 4.2). Any COD used for growth of denitrifiers (and methanogens to a lesser degree) was recycled back into the system upon biomass decay and fermentation.

Acetic and propionic acids were detected with traces of isobutyric, butyric, and isovaleric acids in all nitrate-amended cultures. All VFAs were consumed within 2.4 days of incubation in the nitrate-amended cultures (Figure 4.3). Complete VFA utilization occurred in less time in the 350 mg N/L nitrate-amended culture compared to that of the control culture, which is attributed to the faster utilization of these VFAs by the denitrifiers (Figure 4.2C). VFAs were completely consumed in the 350 mg N/L nitrate-amended culture before the complete reduction of N-oxides, indicating that nitrate reducers utilized electron donors other than VFAs, such as H_2 or monomers (Figures 4.2B and 4.2C). In addition, the absence of acetate before the onset of methanogenesis suggests that methane was produced mainly from H_2/CO_2 in the 350 mg N/L nitrate-amended culture (Figures 4.2A and 4.2C). There was no significant difference in the VFA production and consumption profiles in the control and the nitrate-amended cultures (Figures 4.2C and 4.3), indicating that nitrate reduction did not affect the fermentation

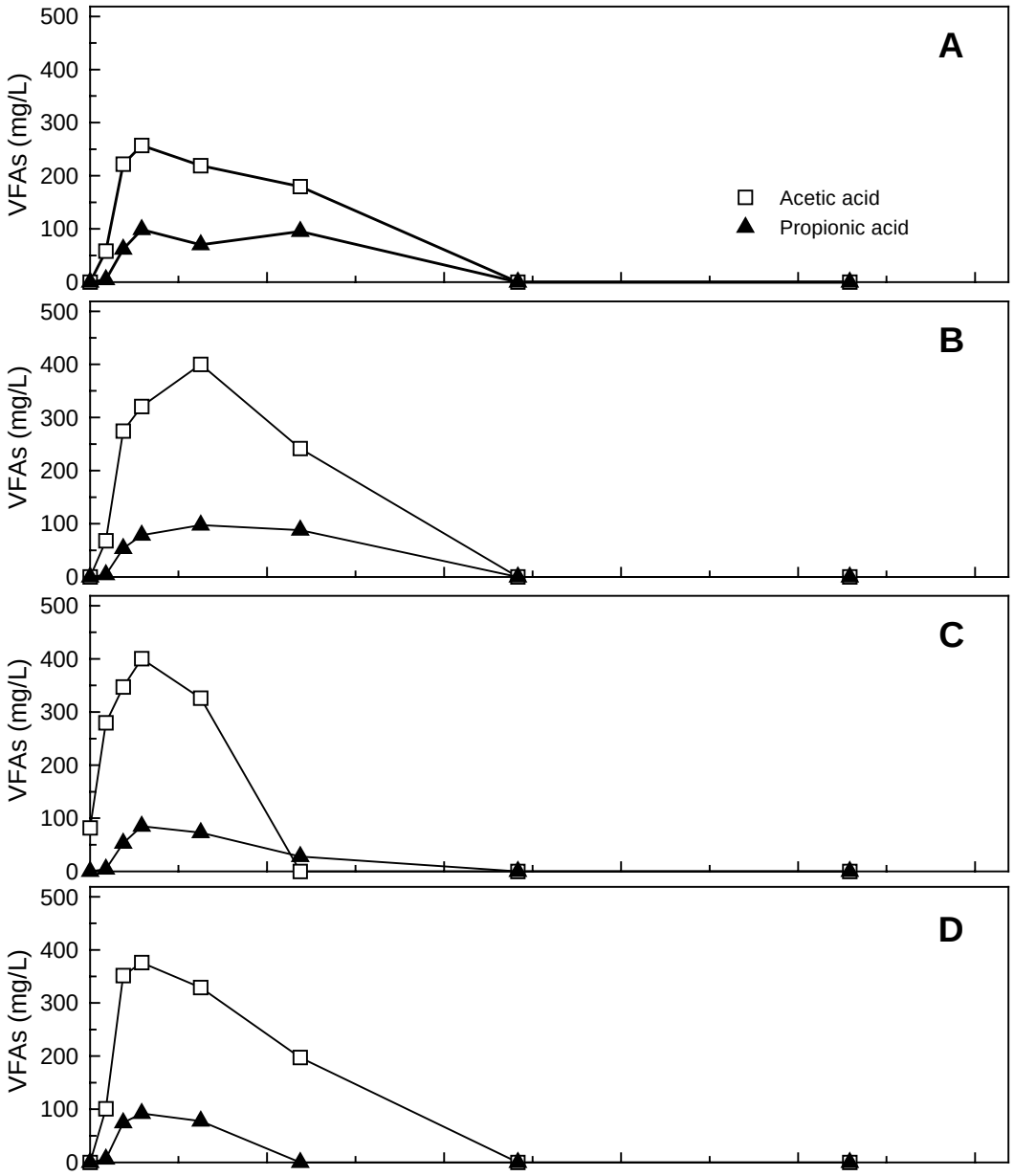

Figure 4.3. Effect of nitrate on the sulfide-free mixed methanogenic culture. VFA production and consumption profiles in the (A) control, (B) 10 mg N/L, (C) 75 mg N/L, (D) 150 mg N/L nitrate-amended cultures.

process. Klüber and Conrad (1998b) also reported that the addition of nitrate to soil microcosms obtained from a rice paddy field did not influence acetate concentrations. In the present study, the total processed COD in the nitrate-amended cultures was close to that of the control culture indicating that acidogenesis (e.g., fermentation of monomers) was also not affected by nitrate reduction (Table 4.2).

The nitrite inhibition assay lasted for 80 days. Addition of nitrite also resulted in suppression of methanogenesis for all nitrite-amended cultures (Figure 4.4A). Methanogenesis recovered immediately after all N-oxides were reduced in the 17, 50, and 125 mg N/L nitrite-amended cultures (Figure 4.4A and Table 4.2). Similar to the 350 mg N/L nitrate-amended culture, required COD_{total} for N-oxide reduction was higher than COD_{in} in the 500 mg N/L nitrite-amended culture (Table 4.1). However, methane production was also observed in the 500 mg N/L nitrite-amended culture suggesting that biomass decay provided a considerable amount of electron equivalents, enough for both N-oxide reduction and methane production in the nitrate- and nitrite-amended cultures. Recovery of methanogenesis occurred approximately 7 and 10 days after the complete reduction of N-oxides in the 250 and 500 mg N/L nitrite-amended cultures (Table 4.2). Nitric oxide production was observed qualitatively in the chromatograms between 0.3 and 2.8 d of incubation in the 500 mg N/L nitrite-amended culture. A transient decrease in the rate of nitrite reduction was observed at the same time that production of nitric oxide was observed (Figure 4.4B). Nitrite reduction resumed only after the disappearance of nitric oxide peaks. A similar behavior was observed in the 250 mg N/L nitrite-amended culture where nitrite reduction was suppressed by the production of nitric oxide. Schulthess et al. (1995) reported that when a denitrifying activated sludge was saturated

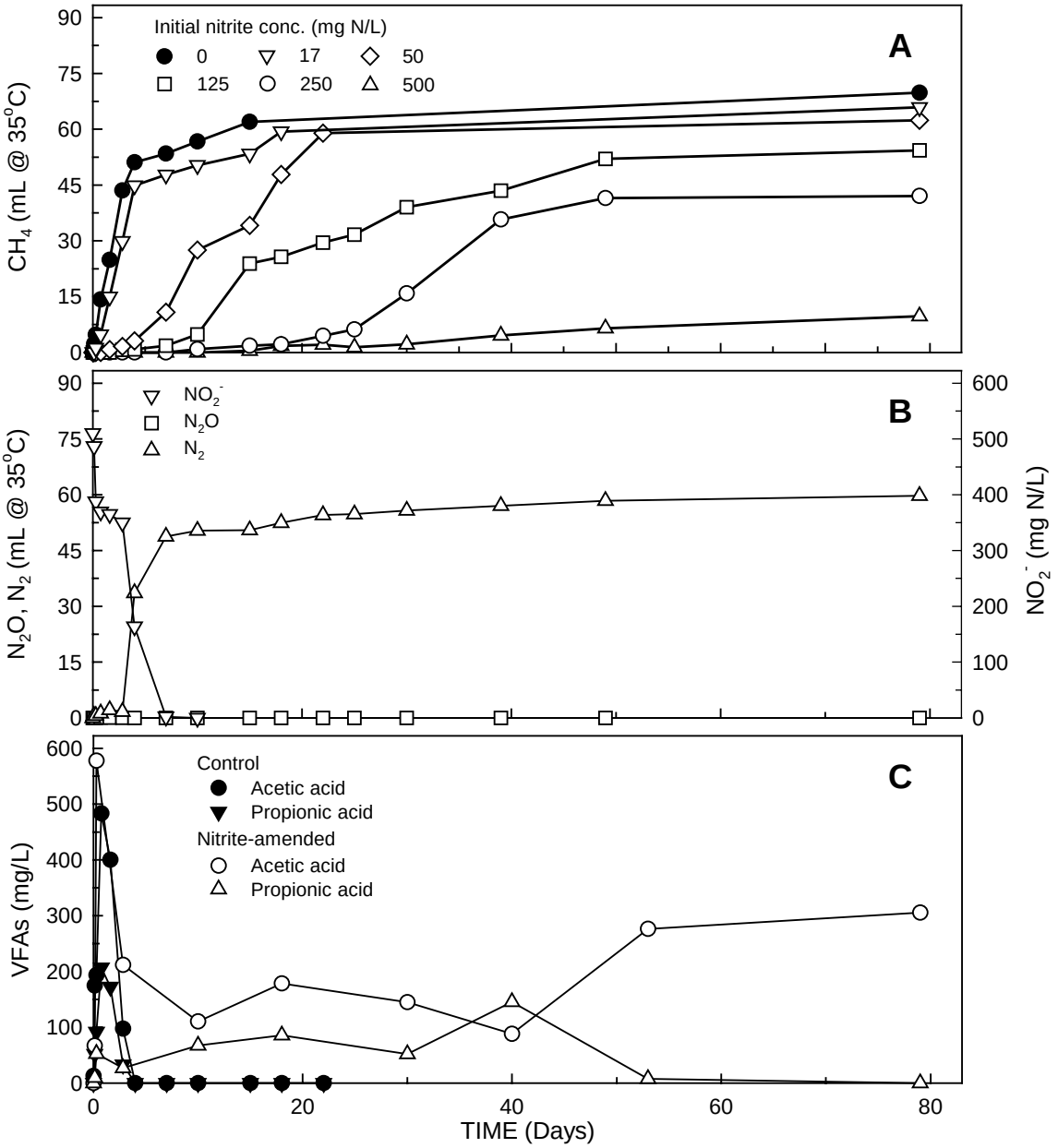

Figure 4.4. Effect of nitrite on the sulfide-free mixed methanogenic culture. (A) Cumulative methane production at different initial nitrite concentrations, (B) production and consumption profiles of N-oxide species in the 500 mg N/L nitrite-amended culture, (C) VFA production and consumption profiles in the control and 500 mg N/L nitrite-amended cultures.

with nitrite, NO accumulation was observed due to inactivation of the nitric oxide reductase by nitrite and consequently, accumulation of NO caused inhibition of nitrate, nitrite, and nitrous oxide reductases. Nitric oxide is a highly reactive radical with an unpaired electron, and is highly toxic to many bacterial species (Zumft, 1993). Therefore, both the relatively slow nitrite reduction and the long delay in methane production in both the 250 and 500 mg N/L nitrite-amended cultures can be attributed to the transient accumulation of nitric oxide. The initial methane production rates in the nitrite-amended cultures were significantly lower compared to that of the control culture (Table 4.2). Although the COD_{nox} requirements were the same in the corresponding nitrate- and nitrite-amended cultures, the methane production rates were lower in the nitrite-amended cultures indicating a higher degree of toxicity of nitrite to the methanogenic culture.

Acetic and propionic acid with traces of butyric acid were detected in all nitrite-amended cultures. In all nitrite-amended cultures, fast production and consumption of VFAs was observed at the beginning of incubation. However, complete reduction of N-oxides resulted in accumulation of VFAs in the 50, 125, 250, and 500 mg N/L nitrite-amended cultures. The VFA production and consumption profile was very similar in the control and the 17 mg N/L nitrite-amended cultures (Figure 4.5A). However, as a result of the increase in the initial nitrite concentration, VFAs (mainly acetic and propionic acid) accumulated for 10, 20, and 40 days in the 50, 125, and 250 mg N/L nitrite-amended cultures (Figure 4.5). Long-term accumulation of acetic and propionic acid was observed in the 500 mg N/L nitrite-amended culture (Figure 4.4C). Recovery of methanogenesis did not have any impact on the acetic acid concentration in the 500 mg N/L nitrite-amended culture suggesting that the main pathway of methane production was

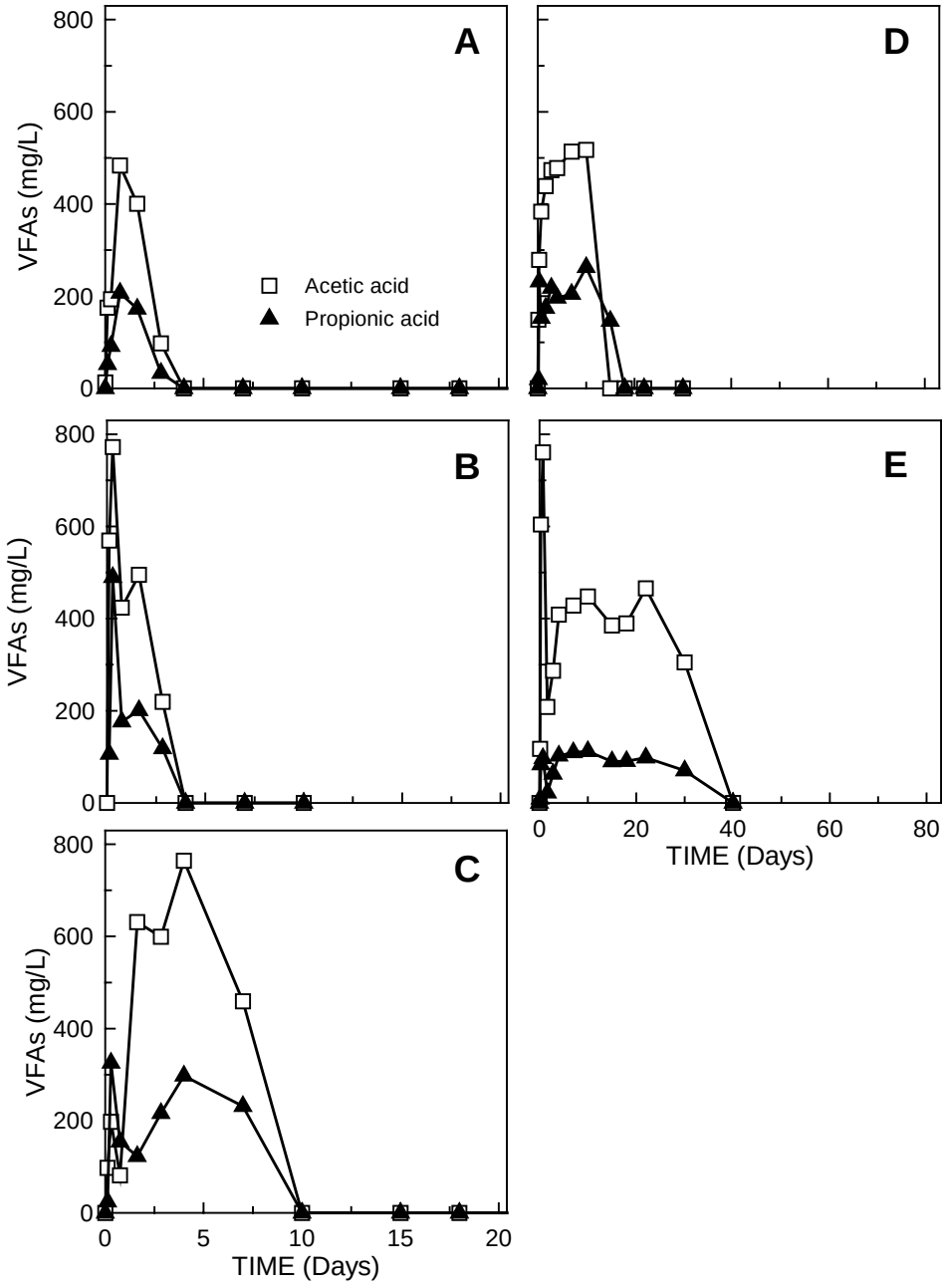

Figure 4.5. Effect of nitrite on the sulfide-free mixed methanogenic culture. VFA production and consumption profiles in the (A) control, (B) 17 mg N/L, (C) 50 mg N/L, (C) 125 mg N/L and (D) 250 mg N/L nitrite-amended cultures.

hydrogenotrophic methanogenesis (Figures 4.4A and 4.4C). Accumulation of propionate was presumably due to inhibition of propionate fermentation by H_2 accumulation. Utilization of H_2 by methanogens decreased the H_2 concentration allowing propionate fermentation to take place, which resulted in an increase of acetic acid concentration in the 500 mg N/L nitrite-amended culture. However, acetic acid was not further utilized most likely due to inhibition of acetoclastic methanogenesis and approximately 20.7% of the initial COD accumulated as VFA (acetic acid) in the 500 mg N/L nitrite-amended culture at the end of the 80-d incubation period (Table 4.2). Similar to the 500 mg N/L nitrite-amended culture, recovery of methane production did not impact the acetic acid concentration in the 50, 125, and 250 mg N/L nitrite-amended cultures (Figure 4.5). However, concomitant to the decrease in the propionic acid concentration, all acetic acid was utilized by methanogens suggesting that at concentrations lower than 250 mg N/L nitrite, acetoclastic methanogenesis was not completely inhibited. Although most methanogens can grow on H_2 and CO_2, acetate is the major methane precursor in anaerobic digesters and other habitats (e.g., fresh water sediments and soil). Therefore, inhibition of acetoclastic methanogenesis by N-oxides can adversely impact methane production in both natural and engineered systems. Inhibition of acetoclastic methanogenesis due to the addition of N-oxides in rice field soil slurries has been reported (Klüber and Conrad, 1998b). The total processed COD in the 17, 50, and 125 mg N/L nitrite-amended cultures was very close to that of the control culture (Table 4.2). However, the total COD processed in the 250 and 500 mg N/L nitrite-amended cultures was lower compared to that of the control culture indicating that nitrite reduction had an

inhibitory effect on the fermentation and acidogenesis processes leading to accumulation of unprocessed COD at the end of the incubation.

The nitric oxide inhibition assay lasted for 34 days. As mentioned above, due to the high toxicity of nitric oxide, very low nitric oxide concentrations were tested in this assay. Methane production was not suppressed and the initial methane production rate was close to that of the control culture in the 0.02 mg N/L nitric oxide-amended culture (Figure 4.6A and Table 4.2). However, although methane production was not completely suppressed, the initial methane production rate was lower in the 0.16 mg N/L nitric oxide-amended culture compared to that of the control culture (Figure 4.6A and Table 4.2). Contrary to our results, Klüber and Conrad (1998b) reported that an aqueous concentration of 0.1 mg N/L nitric oxide caused complete cessation of methanogenesis for 4 d, yet the methane production rates were similar as in the control culture after the recovery of methanogenesis in rice paddy field soil microcosms. The difference in the results of these two studies could be attributed to the fact that the effect of N-oxides on methanogens depends on both the concentration of N-oxide and the microbial strain (Chidthaisong and Conrad, 2000). Methane production was completely suppressed and did not recover even after the complete reduction of N-oxides in the 0.8 mg N/L nitric oxide-amended culture (Figure 4.6A). A sudden decrease in the NO level and increase in the N_2 level was observed followed by a slow utilization of NO which lasted more than 10 days in the 0.8 mg N/L nitric oxide-amended culture (Figure 4.6B). In addition, accumulation of nitrous oxide was observed during the slow reduction of nitric oxide implying possible inhibition of nitric oxide on the denitrifying bacteria, mainly on the nitrous oxide reductase. VFA production and consumption profiles in the 0.02 and 0.16

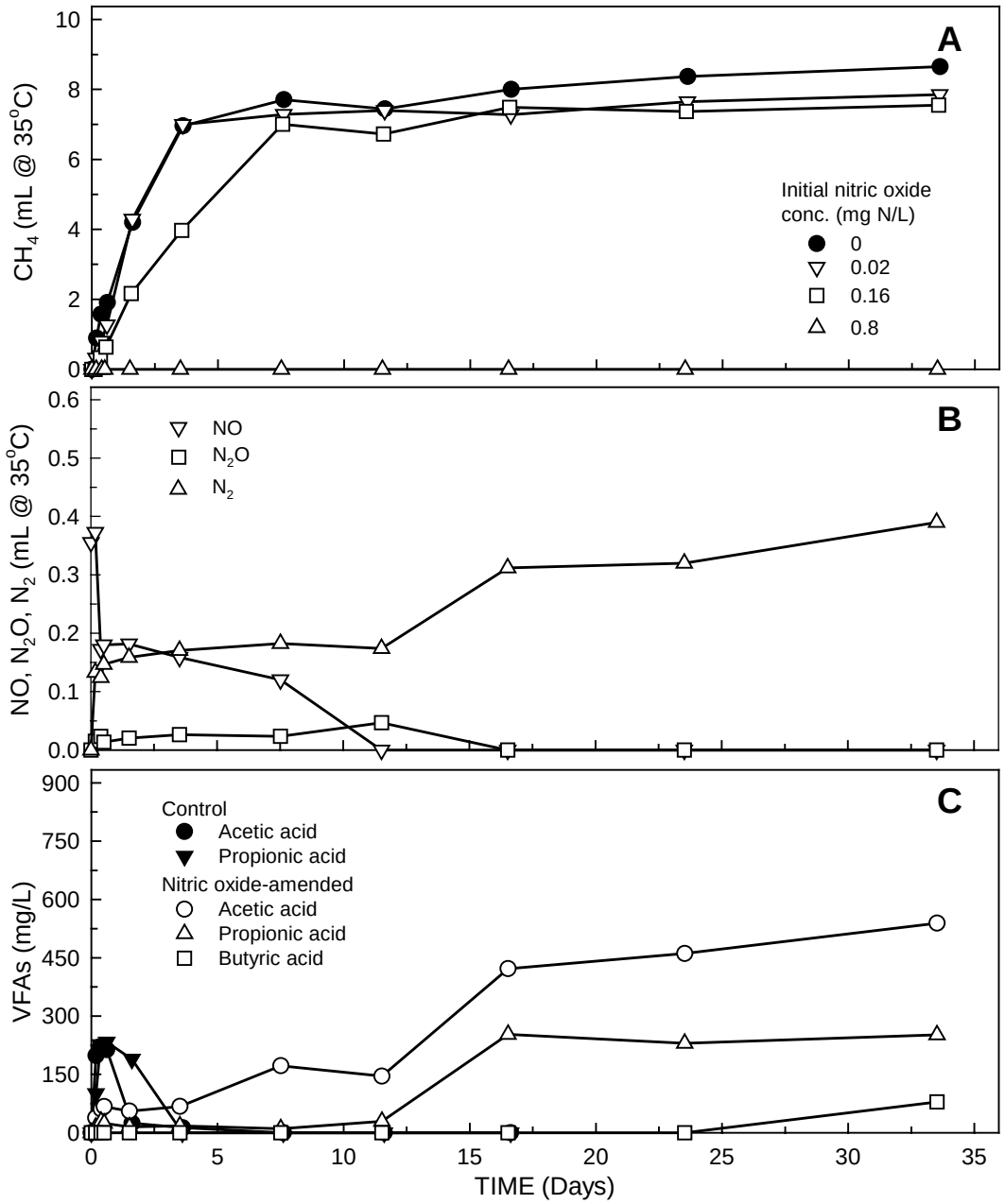

Figure 4.6. Effect of nitric oxide on the sulfide-free mixed methanogenic culture. (A) Cumulative methane production at different initial nitric oxide concentrations, (B) production and consumption profiles of N-oxide species in the 0.8 mg N/L nitric oxide-amended culture, (C) VFA production and consumption profiles in the control and 0.8 mg N/L nitric oxide-amended cultures.

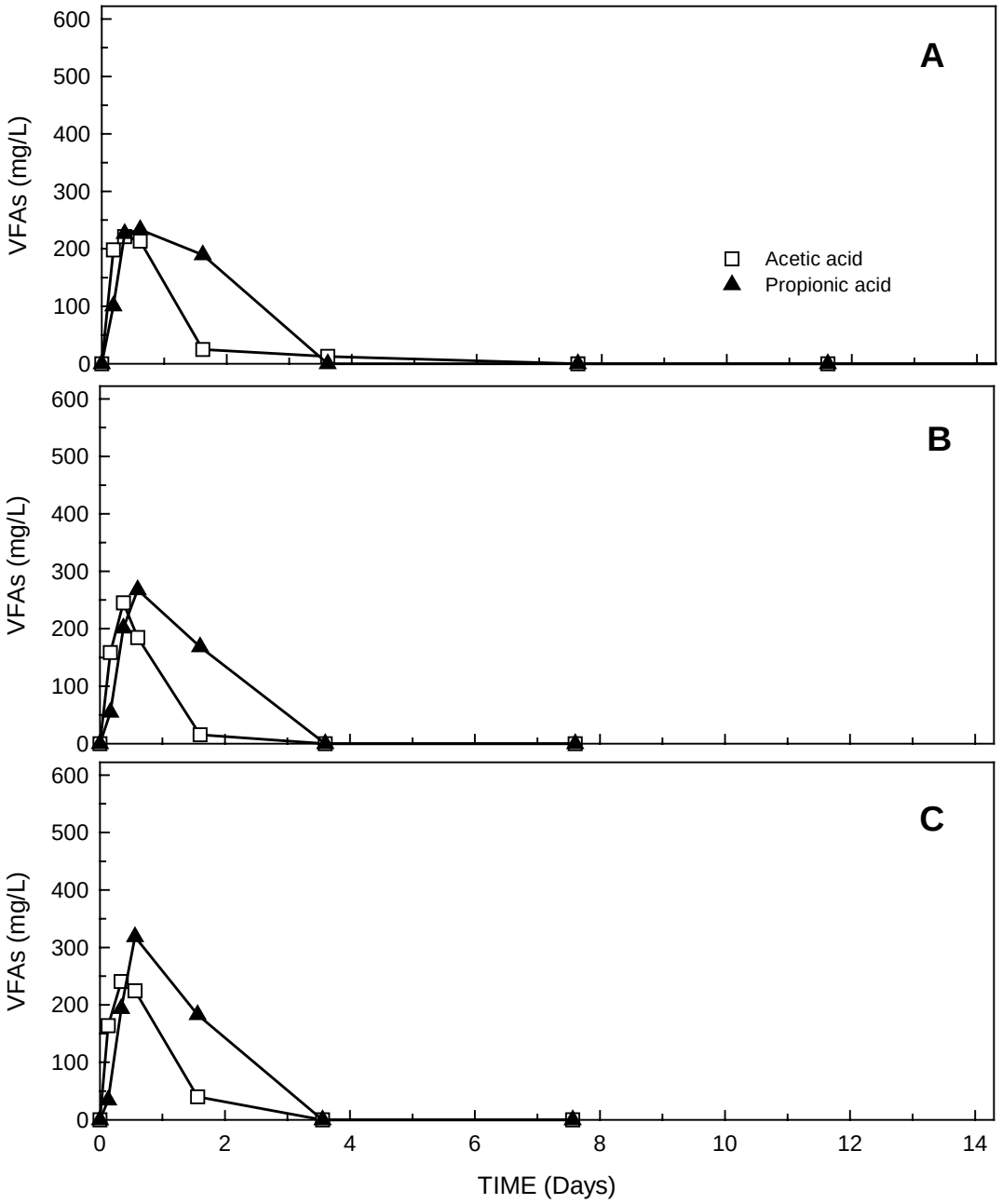

Figure 4.7. Effect of nitric oxide on the sulfide-free mixed methanogenic culture. VFA production and consumption profiles in the (A) control, (B) 0.02 mg N/L, and (C) 0.16 mg N/L nitric oxide-amended cultures.

mg N/L nitric oxide-amended cultures were similar to the control culture (Figure 4.6C and 4.7). However, accumulation of acetic, propionic, and butyric acid was observed in the 0.8 mg N/L nitric oxide-amended culture (Figure 4.6C). Approximately 69% of the total COD remained as VFAs in the 0.8 mg N/L nitric oxide-amended culture at the end of incubation, indicating that both acidogenesis and acetoclastic methanogenesis were inhibited by nitric oxide (Table 4.2).

The nitrous oxide inhibition assay lasted for 19 days. Addition of nitrous oxide resulted in the immediate suppression of methane production for 0.17, 0.9, 1.17, and 1.2 days in the 19, 48, 96, and 191 mg N/L nitrous oxide-amended cultures (Figure 4.8A). In all nitrous oxide-amended cultures, methane production resumed before the complete reduction of nitrous oxide, but the initial methane production rate was lower compared to that of the control culture (Figure 4.8A and Table 4.2). At relatively low concentrations, N_2O was more inhibitory than the equivalent nitrogen concentrations of nitrate and nitrite and less inhibitory than NO. However, at relatively high nitrogen concentrations, nitrate and nitrite caused more inhibition than N_2O, presumably due to the transient accumulation of the intermediate NO at relatively high concentrations. N_2O is directly converted to N_2, thus avoiding the production of NO, which is the most toxic intermediate. It is noteworthy that the initial methane production rate was between 45-50% of that of the control culture regardless of the initial N_2O concentration (Table 4.2). Klüber and Conrad (1998a) reported that inhibition caused by nitrous oxide on both *Methanosarcina barkeri* and *Methanobacterium bryantii* was only partially reversible after the complete removal of the N-oxide, which could be the reason for the relatively slow methane production rate observed in this culture series in the present study. The

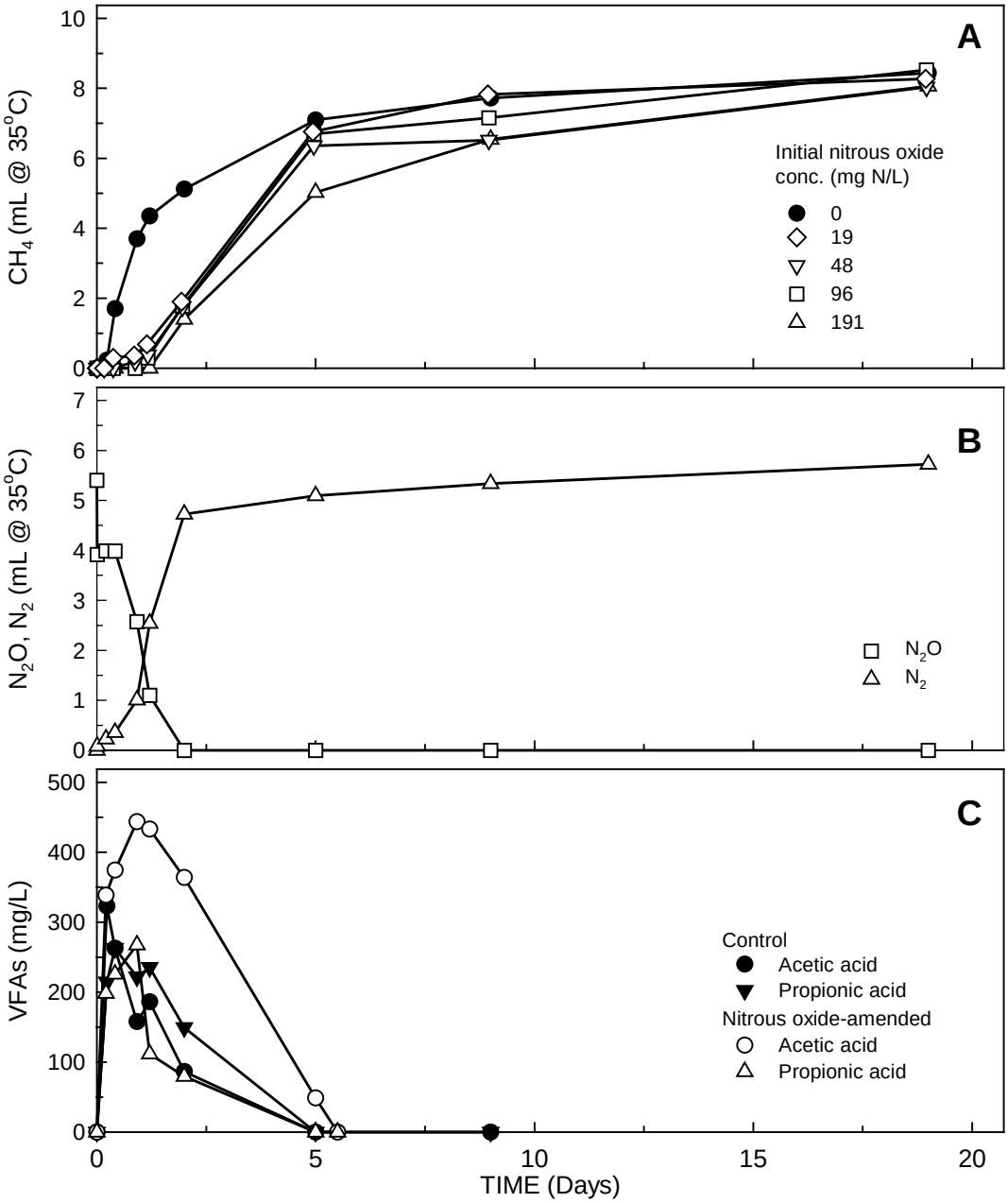

Figure 4.8. Effect of nitrous oxide on the sulfide-free mixed methanogenic culture. (A) Cumulative methane production at different initial nitrous oxide concentrations, (B) production and consumption profiles of N-oxide species in the 191 mg N/L nitrous oxide-amended culture, (C) VFA production and consumption profiles in the control and 191 mg N/L nitrous oxide-amended cultures.

initial fast decrease in the nitrous oxide concentration without a corresponding increase in the N_2 level is attributed to the dissolution of the gas-phase nitrous oxide into the liquid phase in the 191 mg N/L nitrous oxide-amended culture (Figure 4.8B). In all nitrous oxide amended cultures, acetic and propionic acid were the predominant VFAs and their production and consumption rates were similar to those of the control culture as shown for the 191 mg N/L nitrous oxide-amended culture (Figure 4.8C and 4.9). In addition, the total processed COD was very close to that of the control culture indicating that the fermentation of dextrin and peptone was not affected by nitrous oxide at the levels tested in the present study (Table 4.2).

4.3.3. Long-Term Inhibition Assessment in a Sulfide-Free Culture

In this assay, the long-term effect of nitrate reduction on the fermentation of dextrin and peptone and methanogenesis was investigated in four sequential feeding cycles. The incubation period in each cycle was 21, 41, 15, and 31 days, respectively. The pH values of the control culture in each feeding cycle ranged between 6.9 and 7.2. The test culture was amended with 300 mg N/L nitrate only in the second and fourth feeding cycles. The initial/final pH values of the test culture were 6.95/7.01, 7.59/7.39, 7.46/7.14, and 7.26/7.40 at each feeding cycle, respectively. At the end of each feeding cycle, culture media were added to both cultures to compensate for sample withdrawal, which caused an increase in the pH values at the beginning of each subsequent cycle. Differences in methane production were observed in the four feeding cycles of the control culture (Figure 4.10A). Dextrin obtained from a different source was used in the second feeding cycle, which resulted in a higher extent of methane production. Therefore,

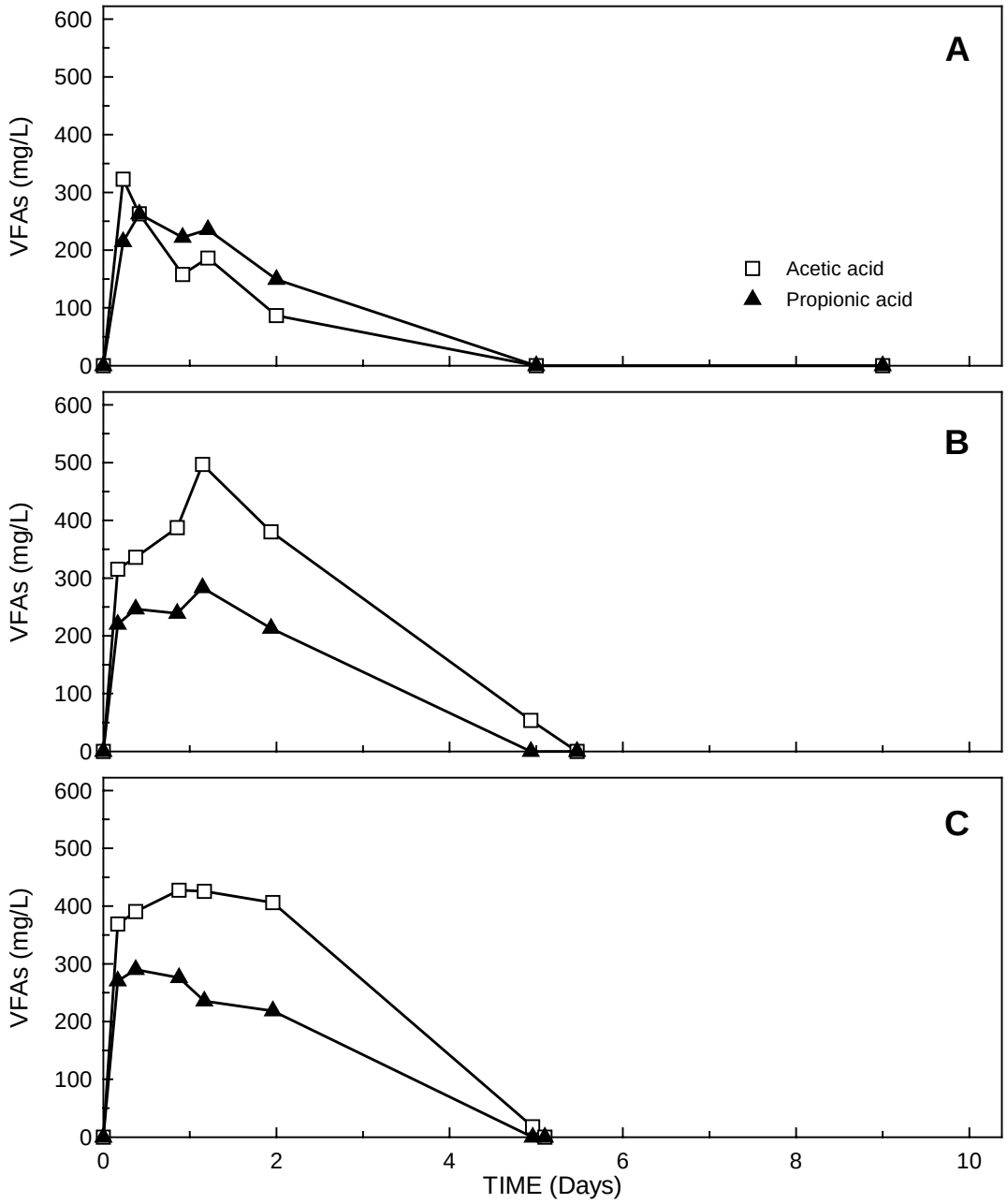

Figure 4.9. Effect of nitrous oxide on the sulfide-free mixed methanogenic culture. VFA production and consumption profiles in the (A) control, (B) 19 mg N/L, and (C) 48 mg N/L nitrous oxide-amended cultures.

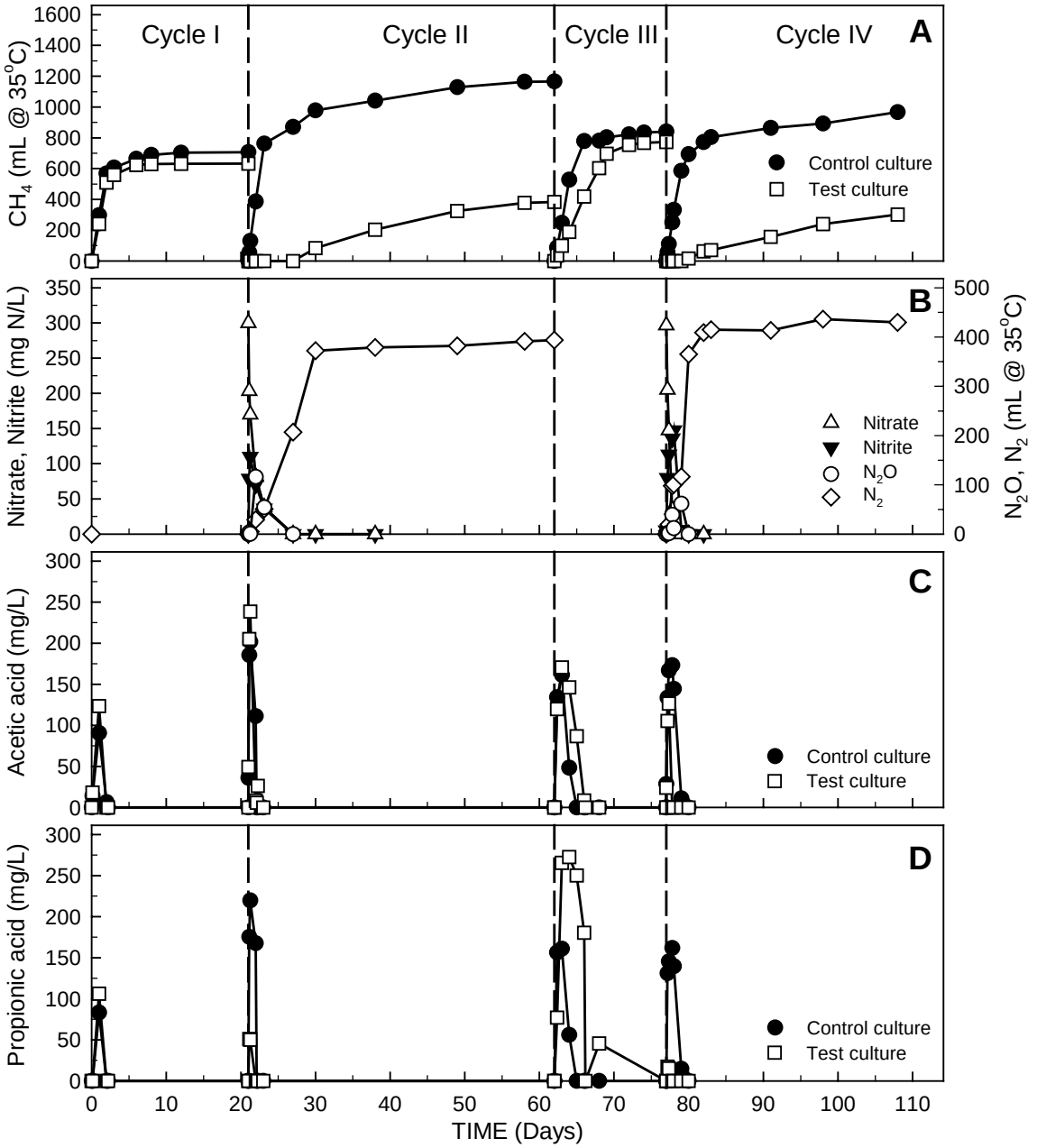

Figure 4.10. Long-term effect of nitrate on the sulfide-free mixed methanogenic culture. (A) Methane, (B) N-oxides and N_2, (C) acetic acid, and (D) propionic acid profiles in the control and test (300 mg N/L nitrate-amended) culture.

each cycle should be considered separately. Initial methane production rates were calculated using linear regression and the results are summarized in Table 4.3. During the first cycle, the methane production rate and extent along with the acetic and propionic acid profiles were comparable in the control and test cultures (Figure 4.10 and Table 4.3). Addition of nitrate to the test culture in the second and fourth feeding cycles caused an immediate cessation of methanogenesis. Complete nitrate and nitrite reduction occurred in 6 d in the second feeding cycle, whereas, nitrate and nitrite reduction occurred within 1 and 2 d, respectively, in the fourth feeding cycle (Figure 4.10B). The observed increased nitrate and nitrite reduction rate in the fourth cycle may be attributed to acclimation of the denitrifying species and/or an increase in their population size. Methane production recovered concomitant to the complete utilization of N-oxides in the second and fourth feeding cycles (Figures 4.10A and 4.10B). As a result of faster N-oxide utilization in the fourth feeding cycle, methane production recovered faster in the test culture than in the second feeding cycle. However, the rate of initial methane production was lower in the fourth feeding cycle compared to that in the second feeding cycle (Table 4.3). In the second and fourth feeding cycles, addition of nitrate did not change the acetic acid concentration, but faster propionic acid utilization was observed in the test culture compared to that of the control culture suggesting that propionic acid may have been preferentially utilized by the denitrifying species (Figures 4.10C and 4.10D). In addition, acetic acid was completely consumed by the denitrifiers and was not detected before or during the methane production in the second and fourth feeding cycles suggesting that the methane was produced mainly from H_2 and CO_2 (Figure 4.10C). It is noteworthy that in the third feeding cycle, although the test culture was not amended with nitrate, the

Table 4.3. Initial methane production rate in the control and nitrate-amended cultures in different feeding cycles of the long-term inhibition assay.

Culture series		Methane production rate (mL/d)[a]	r^2
Cycle I			
	Control culture	284.3 ± 8.8	0.999
	Test culture	254.7 ± 10.2	0.998
Cycle II			
	Control culture	230.9 ± 40.4	0.914
	Test culture[b]	25.3 ± 1.4	0.997
Cycle III			
	Control culture	264.8 ± 9.9	0.997
	Test culture	100.7 ± 1.4	0.998
Cycle IV			
	Control culture	293.9 ± 13.3	0.992
	Test culture[b]	12.2 ± 1.1	0.968

[a] Mean ± standard deviation ($n \geq 3$).
[b] Test culture amended with 300 mg N/L nitrate

methane production rate was approximately 2.6-fold lower than that of the control culture (Figure 4.10A and Table 4.3). The acetic acid production and consumption profile of the test culture was comparable to that of the control culture in the third feeding cycle suggesting that acetoclastic methanogenesis was not affected by the nitrate reduction processes (Figure 4.10C). The slower methane production in the test culture could be related to slow H_2 production as a result of a slower propionic acid utilization as compared to the control culture.

It should be noted that, minimal culture wastage took place throughout this assay. Therefore, both the slow rate of methane production and the accumulation of propionic acid in the test culture during the third feeding cycle may be indicative of changes in the relative culture composition (methanogenic, fermentative, denitrifying, and other sub-populations) as a result of inhibition of methanogenic species. Scheid et al. (2003) reported that the activities of both *Methanosarcina* spp. (acetoclastic) and hydrogenotrophic rice cluster I/Methanomicrobiaceae was suppressed during nitrate reduction in rice root microcosm studies.

4.3.4. Long-Term Inhibition Assessment in a Sulfide-Acclimated Culture

In the first assay, the long-term effect of nitrate reduction on the fermentation of dextrin and peptone and methanogenesis was investigated in six sequential weekly feedings. The incubation period in each cycle was 7 days except for the sixth feeding cycle, which was extended to 80 days to monitor any possible recovery of the nitrate-amended cultures. The pH values of the control culture in each feeding cycle ranged between 7.0 and 7.2. The pH values of the low- and high-level cultures in each cycle

ranged from 6.9 to 7.3 and from 7.1 to 7.4, respectively. Similar levels of methane production were observed in all three cultures in the first feeding cycle proving that the cultures had similar activities (Figure 4.11A). Addition of 10 mg N/L nitrate to the two nitrate-amended cultures did not cause suppression of methanogenesis and similar levels of methane was produced in the control and the two nitrate-amended cultures in the second feeding cycle (Figure 4.11A). A fast rate of nitrate reduction was observed with a transient accumulation of nitrite and nitrous oxide in both nitrate-amended cultures in the second cycle (Figures 4.11A and B; Figure 4.12). However, second time exposure of the two nitrate-amended cultures at an initial nitrate concentration of 10 mg N/L resulted in a decrease in both the rate and extent of methane production compared to that of the control culture in the third feeding cycle (Figure 4.11A). However, the rate of nitrate reduction and intermediate accumulation profiles were the same in the nitrate-amended cultures in the third feeding cycle compared to the second feeding cycle (Figure 4.11B; Figure 4.12). The initial nitrate concentration was increased to 25 mg N/L in the high-level nitrate-amended culture in the fourth, fifth and sixth feeding cycles, whereas the initial nitrate concentration was kept at 10 mg N/L in the low-level nitrate-amended culture in all cycles. Both the low-level and high-level nitrate-amended cultures showed low methane production compared to that of the control culture in the fourth feeding cycle (Figure 4.11A). A significantly higher nitrous oxide accumulation was observed in the high-level nitrate-amended culture compared to that of the low-level nitrate-amended culture (Figure 4.11B). Methane production was not observed in the low-level and high-level nitrate-amended cultures and a higher nitrous oxide accumulation was observed in the high-level nitrate-amended culture in the fifth feeding cycle (Figures 4.11A and B).

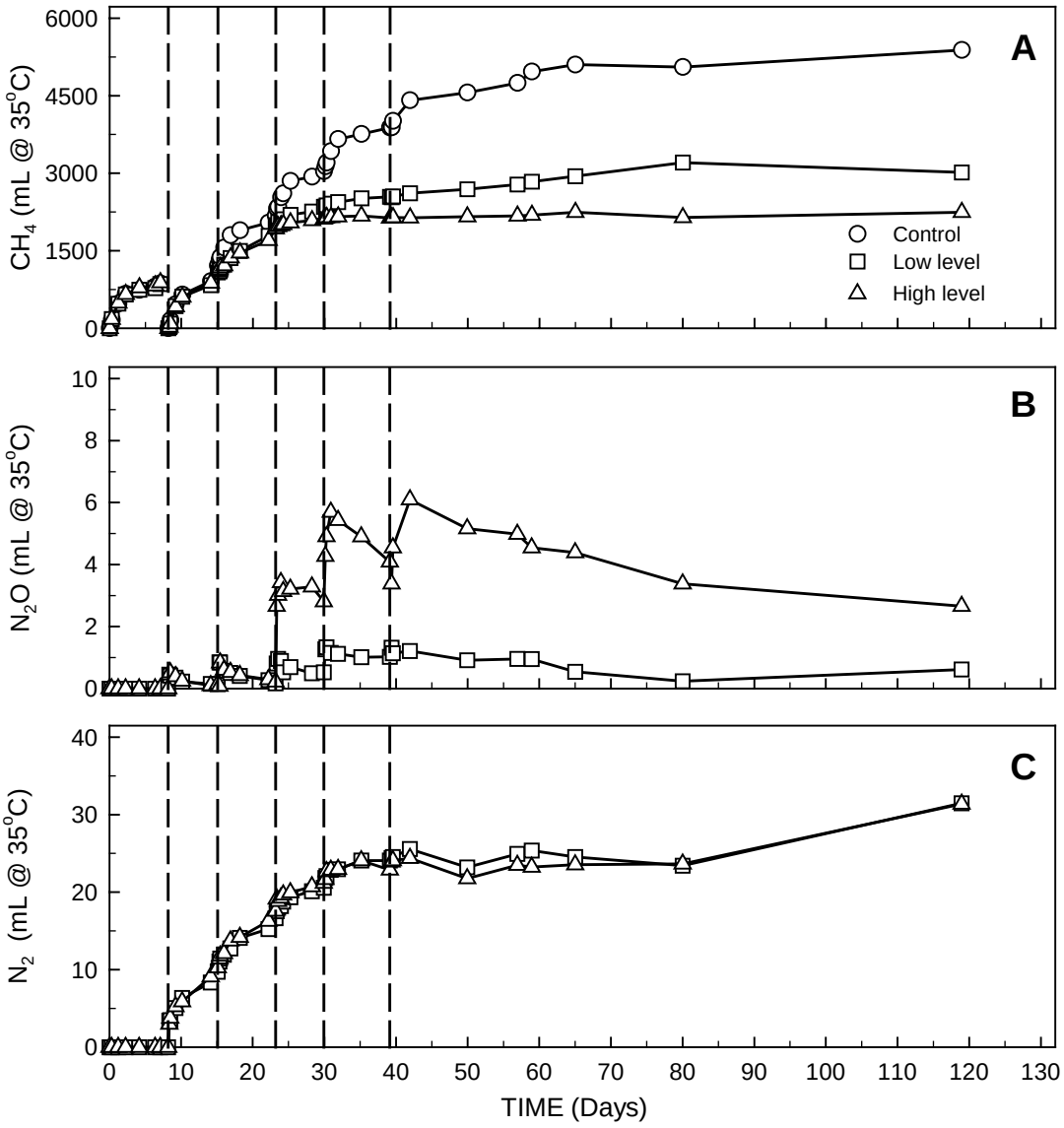

Figure 4.11. Long-term effect of nitrate on a sulfide-acclimated, mixed methanogenic culture as a result of weekly feedings. (A) Methane, (B) nitrous oxide, and (C) nitrogen.

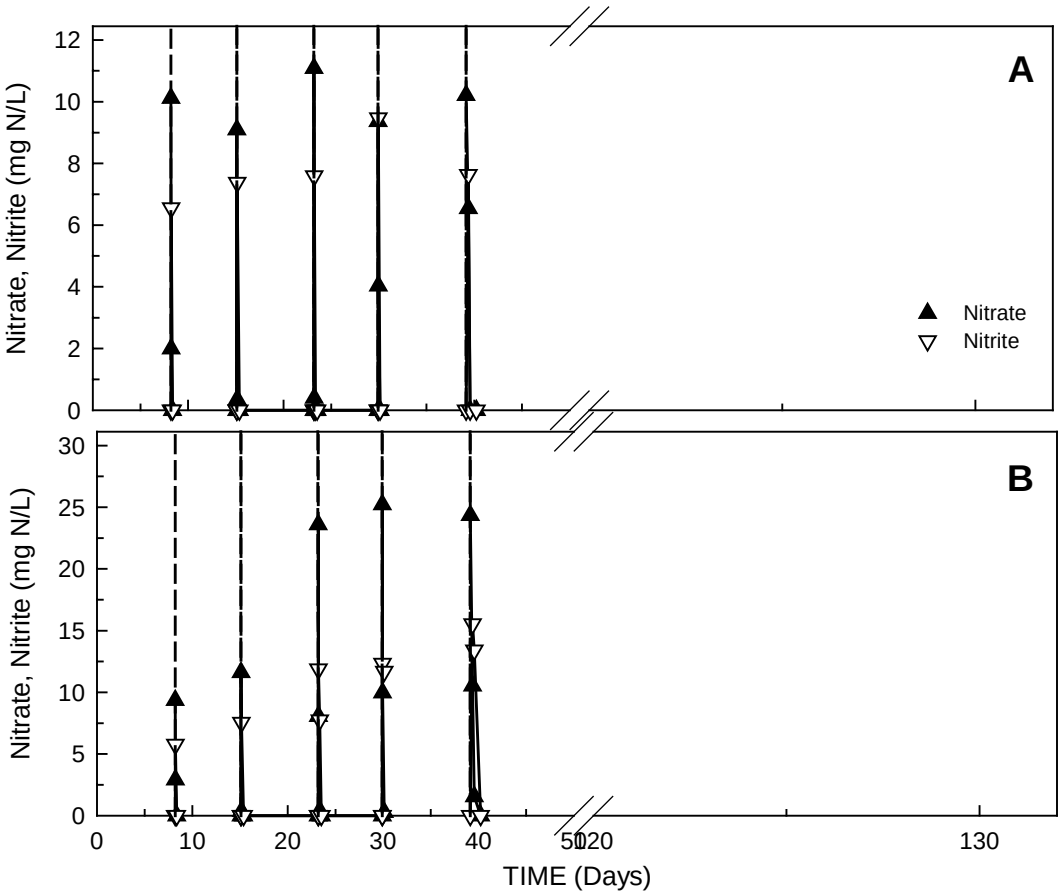

Figure 4.12. Long-term effect of nitrate on a sulfide-acclimated, mixed methanogenic culture as a result of weekly feedings. Nitrate and nitrite profiles in (A) low-level and (B) high-level nitrate-amended cultures.

Similarly, methane production was completely suppressed and did not recover over an incubation period of 80 days in both the low-level and high-level nitrate-amended cultures (Figure 4.11A). Significant nitrous oxide accumulation was observed, which slowly decreased in the sixth feeding cycle (Figure 4.11B). Although fast nitrate reduction and transient nitrite accumulation was observed in the low-level and high-level nitrate-amended cultures in the first five feeding cycles, accumulation of nitrite was observed in both cultures in the sixth feeding cycle (Figures 4.12). Although both nitrate-amended cultures were amended with different concentrations of nitrate in the fourth, fifth, and sixth feeding cycles, the nitrogen gas production was the same as a result of accumulation of nitrous oxide in the high-level nitrate-amended culture (Figure 4.11C). Similar low levels of VFA were observed in all three cultures in the first, second, and third feeding cycles (Figure 4.13). However, accumulation of acetic acid was observed in both the low-level and high-level nitrate-amended cultures in the fourth, fifth, and sixth feeding cycles (Figure 4.13 A). At the end of the sixth feeding cycle, 31% and 23% of the total COD (9000 mg COD/L) remained as acetic acid in the low-level and high-level nitrate-amended cultures, respectively. In addition, accumulation of propionic, isobutyric, and butyric acids was observed in the low-level and high-level nitrate-amended cultures (Figures 4.13). However, isobutyric and butyric acids were completely utilized, whereas 14.5% of total initial COD remained as propionate in the high-level nitrate-amended culture at the end of the sixth feeding cycle. At the end of incubation 100, 56 and 42% of the total COD was converted to methane in the control, low-level, and high-level nitrate-amended cultures, respectively, whereas, 0, 36, and 40% of the total COD remained as total VFAs in the control, low-level, and high-level nitrate-amended cultures,

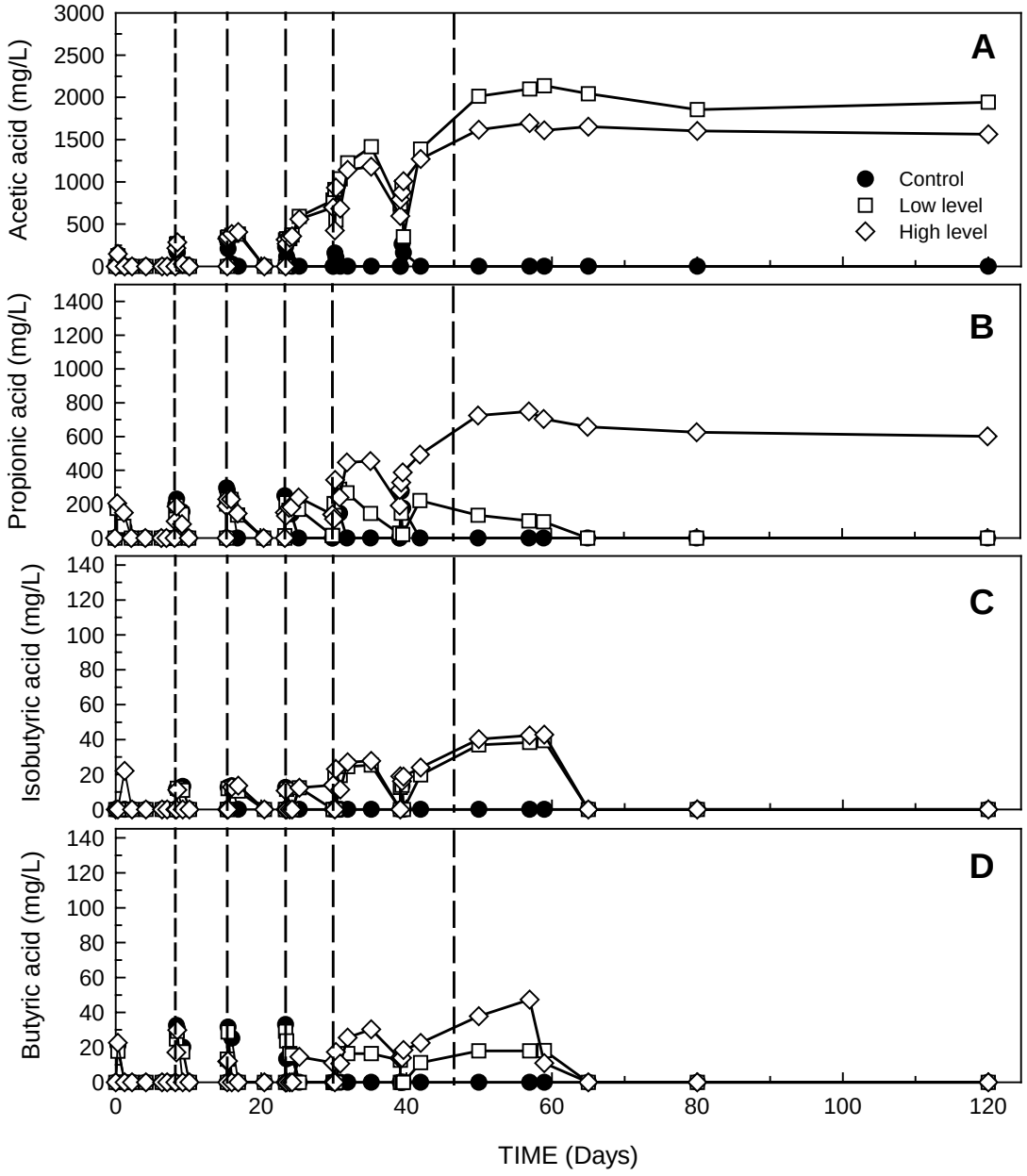

Figure 4.13. Long-term effect of nitrate on a sulfide-acclimated, mixed methanogenic culture as a result of weekly feedings. (A) Acetic acid, (B) propionic acid, (C) isobutyric acid, and (D) butyric acid in the control, low-level, and high-level nitrate-amended cultures.

respectively. In addition, 8 and 19% of the total COD was utilized for nitrate-reduction in the low-level and high-level nitrate-amended cultures, respectively. A prolonged addition of low-level nitrate resulted in inhibition of fermentation and methanogenesis. However, the effect of low-level nitrate addition on fermentation recovered at the end of 120 days of incubation. Repetitive additions of high-level nitrate to the culture resulted in inhibition of both fermentation and methanogenesis and the inhibition did not recover at the end of the incubation.

In the second assay, the long-term effect of nitrate reduction on the fermentation of dextrin and peptone and methanogenesis was investigated in five cycles with sequential daily feedings. The incubation period in each cycle was 7 days. The pH values of the control culture in each feeding cycle ranged between 6.9 and 7.1. The pH values of the low- and high-level cultures in each cycle ranged from 6.9 to 7.2 and from 7.0 to 7.4, respectively. Different from the first assay, methanogenesis was not completely suppressed at the end of 33 days of incubation period in the low-level nitrate-amended culture, probably the result of lower daily amendments of nitrate in the second assay as opposed to higher weekly nitrate-amendments in the first assay (Figure 4.14A). Methane production was significantly suppressed in the high-level nitrate-amended culture starting from the second feeding cycle (Figure 4.14A). Nitrate and nitrite levels could not be monitored due to low daily feedings. Nitrous oxide accumulation was observed in the high-level nitrate-amended culture in each cycle, whereas in the low-level nitrate-amended culture denitrification intermediates were not observed (Figure 4.14B). Acetic and propionic acid accumulation was observed in each cycle and 20% of the total COD (9000 mg COD/L) remained as acetic acid at the end of incubation in the high-level

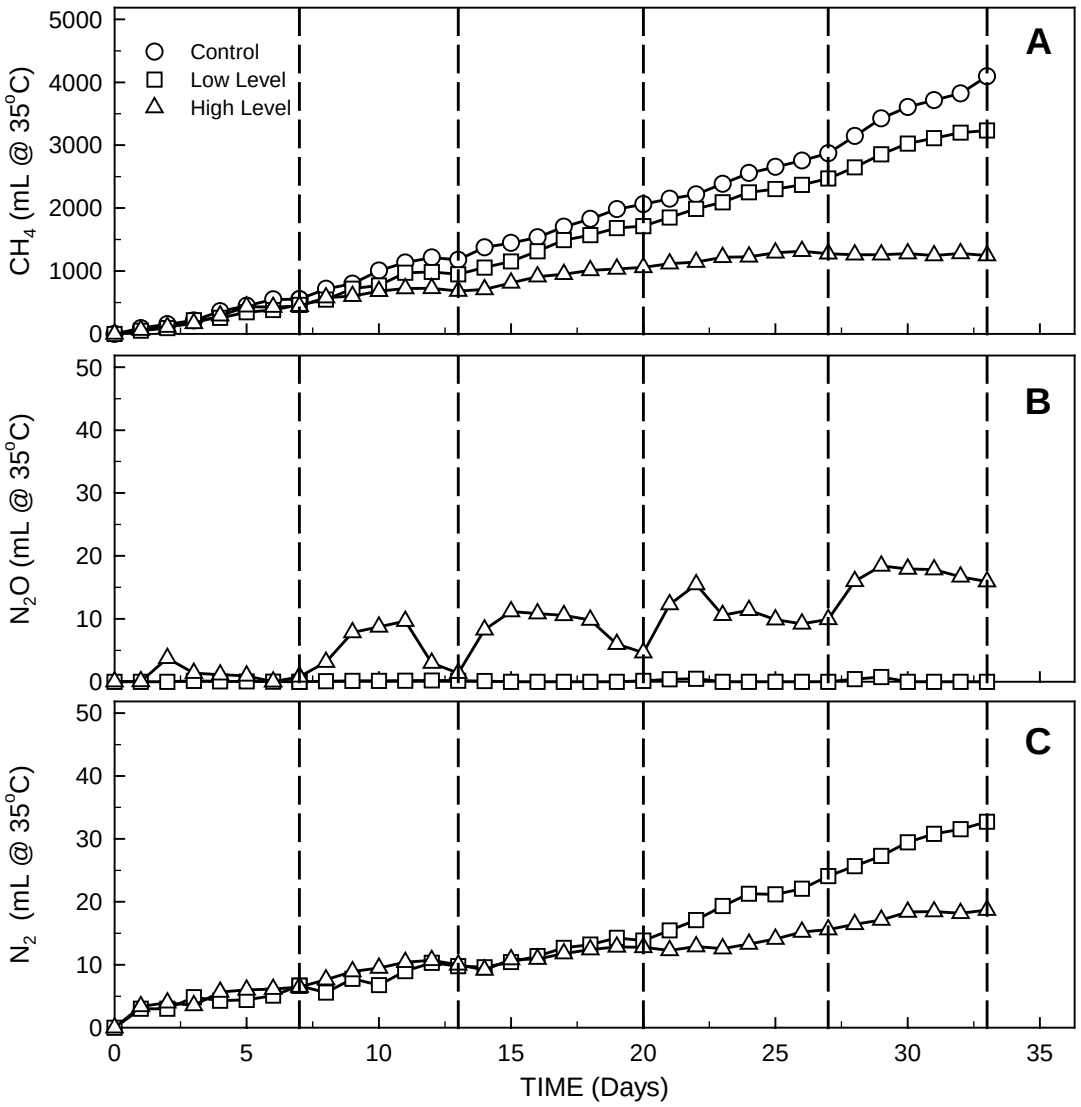

Figure 4.14. Long-term effect of nitrate on a sulfide-acclimated, mixed methanogenic culture as a result of daily feedings. (A) Methane, (B) nitrous oxide, and (C) nitrogen.

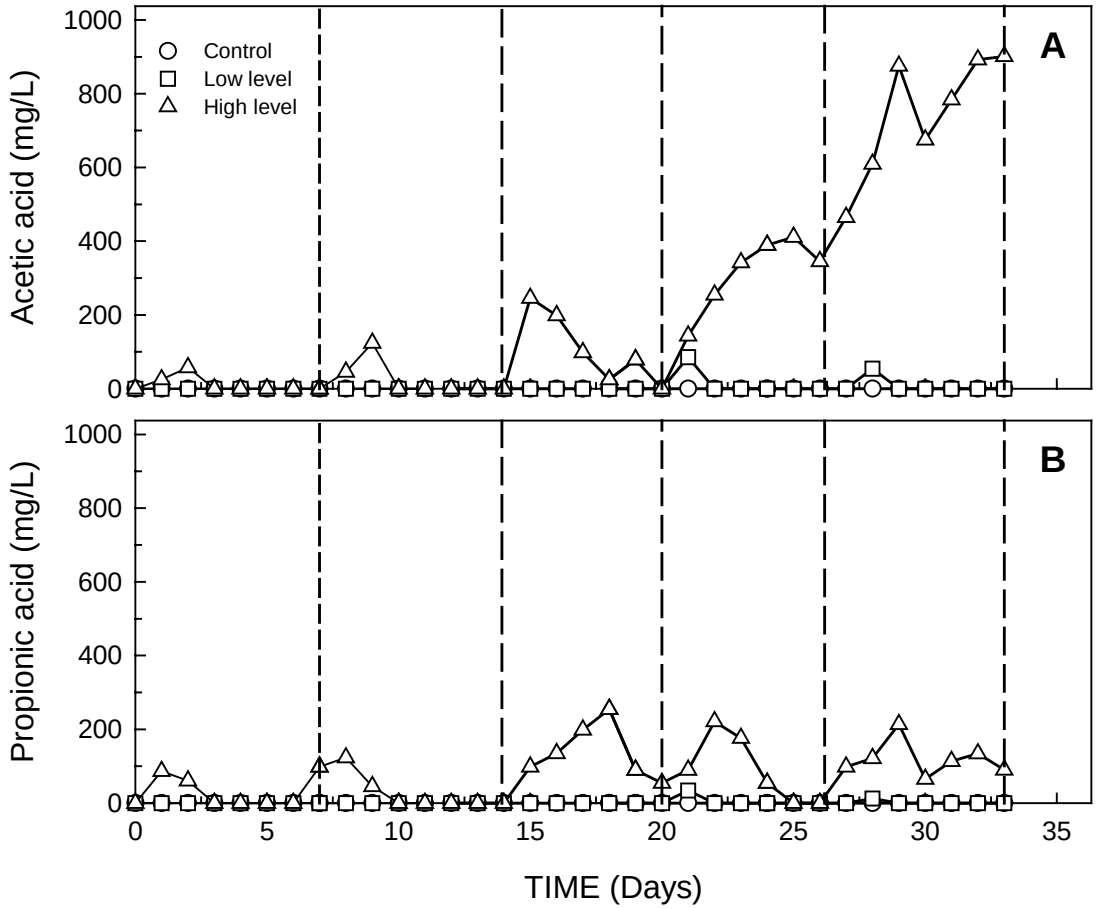

Figure 4.15. Long-term effect of nitrate on a sulfide-acclimated, mixed methanogenic culture as a result of daily feedings. (A) Acetic acid and (B) propionic acid in the control, low-level, and high-level nitrate-amended cultures.

nitrate-amended culture (Figures 4.15A and B). Approximately 100, 79, and 30% of total COD (9000 mg COD/L) was converted to methane in the control, low-level and high-level nitrate-amended cultures at the end of the incubation. Approximately 10 and 18% of total COD was unaccounted for and presumably accumulated as either unutilized or partially degraded COD in both nitrate-amended cultures.

4.4. Summary

The effect of nitrate, nitrite, nitric oxide, and nitrous oxide on a mixed, sulfide-free and sulfide-acclimated mesophilic (35^{o}C) methanogenic cultures was investigated. Short-term inhibition assays were conducted at a concentration range of 10-350 mg N/L nitrate, 17-500 mg N/L nitrite, 0.02-0.8 mg N/L aqueous nitric oxide, and 19-191 mg N/L aqueous nitrous oxide in a sulfide-free methanogenic culture. Simultaneous methane production and N-oxide reduction was observed in 10 and 30 mg N/L nitrate and 0.02 mg N/L aqueous nitric oxide-amended cultures. However, addition of N-oxide resulted in immediate cessation of methanogenesis in all other cultures. Methanogenesis completely recovered subsequent to the complete reduction of N-oxides to nitrogen gas in all N-oxide amended cultures, with the exception of the 500 mg N/L nitrite- and 0.8 mg N/L aqueous nitric oxide-amended cultures. Partial recovery of methanogenesis was observed in the 500 mg N/L nitrite-amended culture in contrast to complete inhibition of methanogenesis in the 0.8 mg N/L aqueous nitric oxide-amended culture. Accumulation of volatile fatty acids was observed in both cultures at the end of the incubation period. Among all N-oxides, nitric oxide exerted the most and nitrate exerted the least inhibitory

effect on the fermentative/methanogenic consortia. The effect of multiple additions of nitrate (300 mg N/L) on the same methanogenic culture was also investigated.

Long-term exposure of the sulfide-free methanogenic culture to nitrate resulted in an increase of N-oxide reduction rates and decrease of methane production rates, which was attributed to changes in the microbial community structure due to nitrate addition. The long-term effect of nitrate on a sulfide-acclimated mixed, methanogenic culture was investigated by weekly and daily additions of nitrate at low and high initial nitrate concentrations. Weekly additions of nitrate resulted in more severe inhibition of both fermentation and methanogenesis as a result of the relatively high initial nitrate concentration that the microorganisms were exposed. However, due to decreased initial levels of nitrate, daily additions resulted in lower impact on fermentation and methanogenesis in the low-level nitrate-amended cultures. However, although the impact was less severe, both fermentation and methanogenesis was affected in the high-level nitrate-amended culture as a result of daily nitrate additions.

CHAPTER 5

EFFECT OF SULFIDE ON NITRATE REDUCTION AND METHANOGENESIS

5.1. Introduction

It has been reported that sulfide in nitrate reducing environments plays an important role as to whether denitrification or DNRA is the predominant nitrate reduction pathway (Myers, 1972; Brunet and Garcia-Gill, 1996; Percheron et al., 1998; Senga et al., 2006). Brunet and Garcia-Gill (1996) reported that at an extremely low free sulfide concentration (1.6 mg S/L), denitrification was the dominant nitrate reduction pathway, whereas at a relatively high free sulfide concentration (32 mg S/L), incomplete denitrification and ammonia production through DNRA was observed in freshwater sediments. At a high free sulfide concentration, nitrate reduction via DNRA took place because of partial inhibition of NO- and strong inhibition of N_2O-reductases by sulfide (Sørensen et al., 1980; Brunet and Garcia-Gill, 1996). In addition, in earlier studies, DNRA was suggested to occur at high COD/N values (Smith, 1982; Akunna et al., 1992). However, later, it was revealed that the type of nitrate reduction pathway was also related to the type of carbon/electron donor source rather than simply the COD/N value (Akunna et al., 1993; Percheron et al., 1998). It has been reported that denitrification prevails against DNRA when volatile fatty acids (VFAs) are the main electron donor and DNRA becomes the predominant nitrate reduction pathway when glycerol and glucose are used regardless of the COD/N value (Akunna et al., 1993).

The inhibitory effects of nitrate and denitrification intermediates in a sulfide-free, mixed methanogenic culture have been previously described in Chapter 4. However, additional challenges arise in sulfide and nitrate containing methanogenic environments. Although the effect of sulfide on nitrate reduction has been studied before, research considering the combined effects of sulfide and nitrate on the inhibition of methanogenesis by N-oxides is lacking. The objectives of the research presented here were to: (a) assess the effect of sulfide on nitrate reduction and methanogenesis in sulfide-free and sulfide-acclimated mixed, mesophilic (35^{o}C) methanogenic cultures; and (b) assess the effect of the COD/N value on the nitrate reduction pathway.

5.2. Materials and Methods

5.2.1. Sulfide-Free and Sulfide-Acclimated Enriched Methanogenic Cultures

Assays were conducted using two mixed, methanogenic cultures: a *sulfide-free culture* enriched in sulfide-free media, and a *sulfide-acclimated culture* enriched in sulfide-bearing media. Steady-state pH values of the sulfide-free and sulfide-acclimated cultures were 6.9 ± 0.1 and 7.1 ± 0.1 (mean ± standard deviation), respectively. Both cultures were developed with an inoculum obtained from a mesophilic (35^{o}C), municipal anaerobic digester, and were fed with dextrin/peptone (4 g/L dextrin, 2 g/L peptone in the feed solution) and nutrient media (see Chapter 3). The peptone used (Sigma-Aldrich) contains 8% nitrogen and degradation of peptone may yield a maximum of 32 mg NH_4^+-N/L in every culture feeding. In addition, ammonia contribution of the media is 130.2 mg NH_4^+-N/L. Therefore, the total amount of ammonia supplied by the feed and media was 194 mg NH_4^+-N/L when the culture was fed twice a week. The total sulfide concentration

provided by the trace metals and vitamins in the media was 0.2 mg S/L. Therefore, the total amount of sulfide in the sulfide-free and sulfide-amended media was 0.2 and 67 mg S/L, respectively. The two methanogenic cultures (6 L liquid volume each) were maintained at 35ºC and were fed twice a week with a hydraulic (and solids) retention time of 35 days and were continuously mixed using a magnetic stirrer. Both cultures were maintained under the above-stated conditions for over two years and three years before the initiation of this study, respectively. The steady-state gas-phase CH_4 and CO_2 and the total solids (TS) and volatile solids (VS) concentrations of the sulfide-free culture were 59.7± 0.8%, 40.3 ± 0.9%, 5,524 ± 426 mg/L, and 1,810 ± 84 mg/L (mean ± standard deviation), respectively. Likewise, the steady-state gas-phase CH_4 and CO_2 and the TS and VS concentrations of the sulfide-acclimated culture were 60.7 ± 0.8%, 39.2 ± 0.4%, 6,900 ± 300 mg/L, and 2,200 ± 100 mg/L (mean ± standard deviation), respectively.

5.2.2. Abiotic Controls

In order to assess the possibility of abiotic nitrate reduction by sulfide or any other media components, an abiotic control assay was conducted using 160-mL serum bottles (100 mL liquid volume). The serum bottles were sealed with rubber stoppers and aluminum crimps and pre-flushed with helium gas. Five types of autoclaved controls were used: deionized water (DI), sulfide-free culture media, sulfide-amended culture media, sulfide-free culture, and sulfide-acclimated culture. All controls were autoclaved at 121^{o}C for 1 hour twice in two consecutive days and then amended with 40 mg N/L nitrate. Incubation of all controls was carried out in the dark at 35ºC under continuous

mixing conditions magnetic stirrers. All controls were monitored throughout the incubation period for nitrate and possible nitrate reduction products.

5.2.3. Effect of Mixing Assay

A batch assay was conducted to assess the effect of mixing on process interactions between methanogens and nitrate reducers in the presence of sulfide using four 500-mL serum bottles (400 mL liquid volume) sealed with rubber stoppers and aluminum crimps and pre-flushed with helium gas. An aliquot of 200 mL sulfide-free mixed, methanogenic culture and 185 mL culture media were anaerobically transferred to each serum bottle. The cultures were amended with $Na_2S \cdot 9H_2O$ resulting in a total sulfide concentration of 67 mg/L, which is the total sulfide concentration of the sulfide-acclimated methanogenic culture. The cultures were then incubated at 35^{o}C overnight to achieve equilibrium of sulfide species. Then, dextrin/peptone mixture was added to all serum bottles resulting in an initial concentration of 1022/521mg/L (1,091/546 mg COD/L), respectively. Two of these cultures were amended with 300 mg N/L using aliquots of $NaNO_3$ while the other two were used as nitrate-free, control cultures. One control and one nitrate-amended culture were not mixed over the entire incubation period, whereas, the other control and nitrate-amended cultures were continuously mixed during the incubation period using magnetic stirrers. Replicate sacrificial cultures were setup for the explicit measurement of initial pH and biomass concentration. The initial biomass concentration in the serum bottles was 1,084 ± 22 mg VS/L. Incubation was carried out in the dark at 35ºC.

5.2.4. Effect of Sulfide on Nitrate Reduction and Methanogenesis in a Sulfide-Free Enriched Culture

Two batch assays were performed using the sulfide-free enriched culture. In the first assay, four 500-mL serum bottles (400 mL liquid volume) sealed with rubber stoppers and aluminum crimps and pre-flushed with helium gas were used following the above described procedure. The carbon source was dextrin/peptone (1,022/521mg/L; 1,091/546 mg COD/L). Two of these cultures were kept sulfide-free and amended with 0 and 350 mg N/L nitrate serving as control and nitrate-amended cultures, respectively. The other two cultures were amended with 67 mg S/L sulfide and were also amended with 0 and 350 mg N/L nitrate. It should be noted that, throughout this work, sulfide amendments stated are total sulfide doses, i.e., total sulfide added to the culture without taking into account sulfide speciation and partitioning. A relatively low COD/N value (equal to 4.7) was used in this assay. A $NaNO_3$ stock solution was used to prepare the two nitrate-amended cultures. The initial biomass concentration in the serum bottles was 1,167 ± 14 mg VS/L. Incubation of these cultures was carried out in the dark at 35ºC with continuous mixing using magnetic stirrers.

In the second assay, the effect of varying sulfide concentrations on nitrate reduction and methanogenesis was tested using the sulfide-free culture. Twelve 160-mL serum bottles (112 mL liquid volume) sealed with rubber stoppers and aluminum crimps and pre-flushed with helium gas were used. An aliquot of 70 mL mixed, sulfide-free methanogenic culture and 20 mL culture media were anaerobically transferred to each serum bottle, and a dextrin/peptone mixture was added resulting in an initial concentration of 1,022/521mg/L (1,091/546 mg COD/L), respectively. In order to make

sure that the added amount of sulfide did not have any inhibitory effect on the fermentative/methanogenic consortia, six cultures were amended with 0, 10, 20, 40, 80, and 100 mg S/L using different aliquots of a $Na_2S \cdot 9H_2O$ stock solution. Another six cultures were amended with the above-stated sulfide concentrations and an aliquot of a $NaNO_3$ stock solution was added to result in an initial nitrate concentration of 120 mg N/L. The COD/N value for this assay was equal to 13.6. After the addition of sulfide, all cultures were incubated at 35°C overnight in order to achieve equilibrium of sulfide species and then the nitrate and carbon source solutions were added. The initial biomass concentration in the serum bottles was 1,220 ± 5 mg VS/L. Incubation was carried out in the dark at 35ºC with continuous mixing using magnetic stirrers.

5.2.5. Effect of Nitrate on Sulfide-Amended and Sulfide-Acclimated Cultures

A batch assay was conducted to test the effect of nitrate in sulfide-amended and sulfide-acclimated cultures using four 160-mL serum bottles (112 mL liquid volume) sealed with rubber stoppers and aluminum crimps and pre-flushed with helium gas. Aliquots of 70 mL sulfide-free culture and 20 mL culture media were anaerobically transferred to two of these serum bottles and aliquots of $Na_2S \cdot 9H_2O$ were added resulting in a total sulfide concentration of 67 mg S/L. The cultures were continuously mixed overnight in order to achieve equilibrium of sulfide species. These cultures were then amended with 0 (control) and 75 mg N/L nitrate by the addition of aliquots of a $NaNO_3$ stock solution. The other two serum bottles were filled with 70 mL sulfide-acclimated culture and were amended with 0 (control) and 75 mg N/L nitrate. An aliquot of a dextrin/peptone mixture was added to all four serum bottles resulting in an initial

concentration of 952/476 mg/L (1,016/498 mg COD/L), respectively, in all serum bottles. The COD/N value for this assay was equal to 20.2, a relatively higher value than that used in the very first assay in which the effect of sulfide on nitrate reduction and methanogenesis was tested using the sulfide-free enriched culture. The initial biomass concentration in the serum bottles was 1,174 ± 36 mg VS/L.

5.2.6. Effect of COD/N Value on Nitrate Reduction in a Sulfide-Acclimated Culture

A batch assay was conducted to test the effect of sulfide on nitrate reduction and methanogenesis using four 160-mL serum bottles (100 mL liquid volume) sealed with rubber stoppers and aluminum crimps and pre-flushed with helium gas. An aliquot of 70 mL sulfide-acclimated methanogenic culture and 20 mL culture media were anaerobically transferred to each serum bottle. Dextrin was used as the sole carbon source in this assay in order to eliminate ammonia production as a result of peptone degradation, thus facilitating DNRA assessment. Aliquots of a dextrin solution were added to each bottle resulting in an initial concentration of 468, 937, and 2,812 mg dextrin/L, corresponding to 500, 1000, and 3000 mg COD/L, respectively. An aliquot of $NaNO_3$ stock solution was added to each serum bottle resulting in an initial nitrate concentration of 50 mg N/L. Based on the initial COD and nitrate amendments, the initial COD/N values were equal to 10, 20, and 60. The initial biomass concentration in the serum bottles was 1,089 ± 21 mg VS/L. Incubation was carried out in the dark at 35ºC with continuous mixing using magnetic stirrers.

5.3. Results and Discussion

5.3.1. Assessment of Abiotic Nitrate Reduction

This assay lasted for 20 days and the initial and final pH values ranged from 7.3 to 7.5. In all autoclaved controls (DI, sulfide-amended media, and sulfide-acclimated culture), a slight decrease ($\leq$ 10%) in the nitrate concentration occurred at the beginning of the incubation (i.e., 3 h), but the nitrate concentration remained the same during the incubation period regardless of the presence of sulfide (Figure 5.1). Nitrite was not detected in any of the autoclaved controls. Likewise, gas production was not observed in the autoclaved controls. Therefore, because nitrate reduction did not occur in any of the autoclaved controls, it is concluded that nitrate reduction in active sulfide-acclimated cultures reported below was biologically mediated.

5.3.2. Assessment of Mixing Significance

This assay, which examined the effect of mixing on sulfide inhibition lasted for 10 days. The initial pH value was 7.4 ± 0.1 (mean ± standard deviation) and the final pH values were 7.2, 7.3, 7.7, and 7.8 for the control without mixing, control with mixing, nitrate-amended without mixing, and nitrate-amended with mixing cultures, respectively. Regardless of amendment, the pH was higher in the continuously mixed cultures than the cultures without mixing. The methane production in the two nitrate-free, control cultures with and without mixing is shown in Figure 5.2A. The initial rate of methane production was 62.2 ± 5.8 and 47.3 ± 2.3 mL/day in the nitrate-free, control cultures with and without mixing, respectively. Therefore, a lower rate of initial methane production was

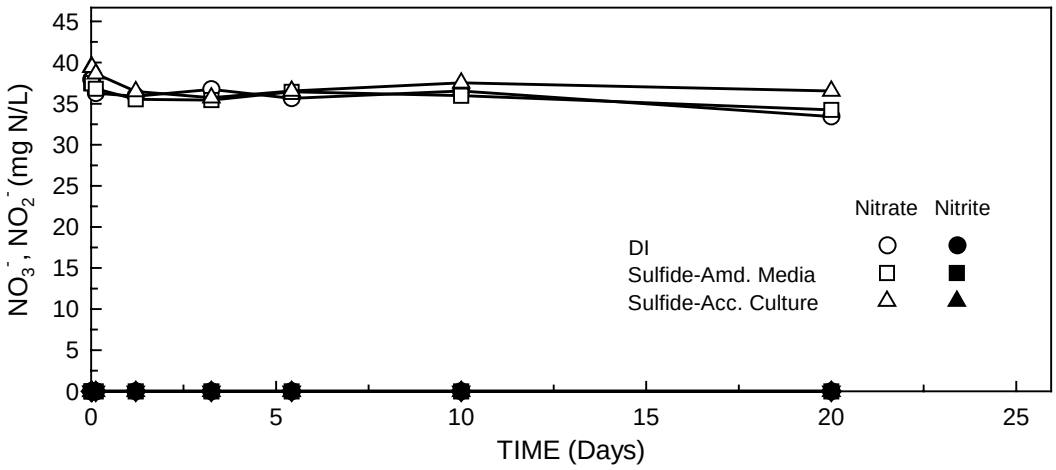

Figure 5.1. Stability of nitrate in autoclaved DI water, sulfide-amended media and sulfide-acclimated culture (abiotic controls).

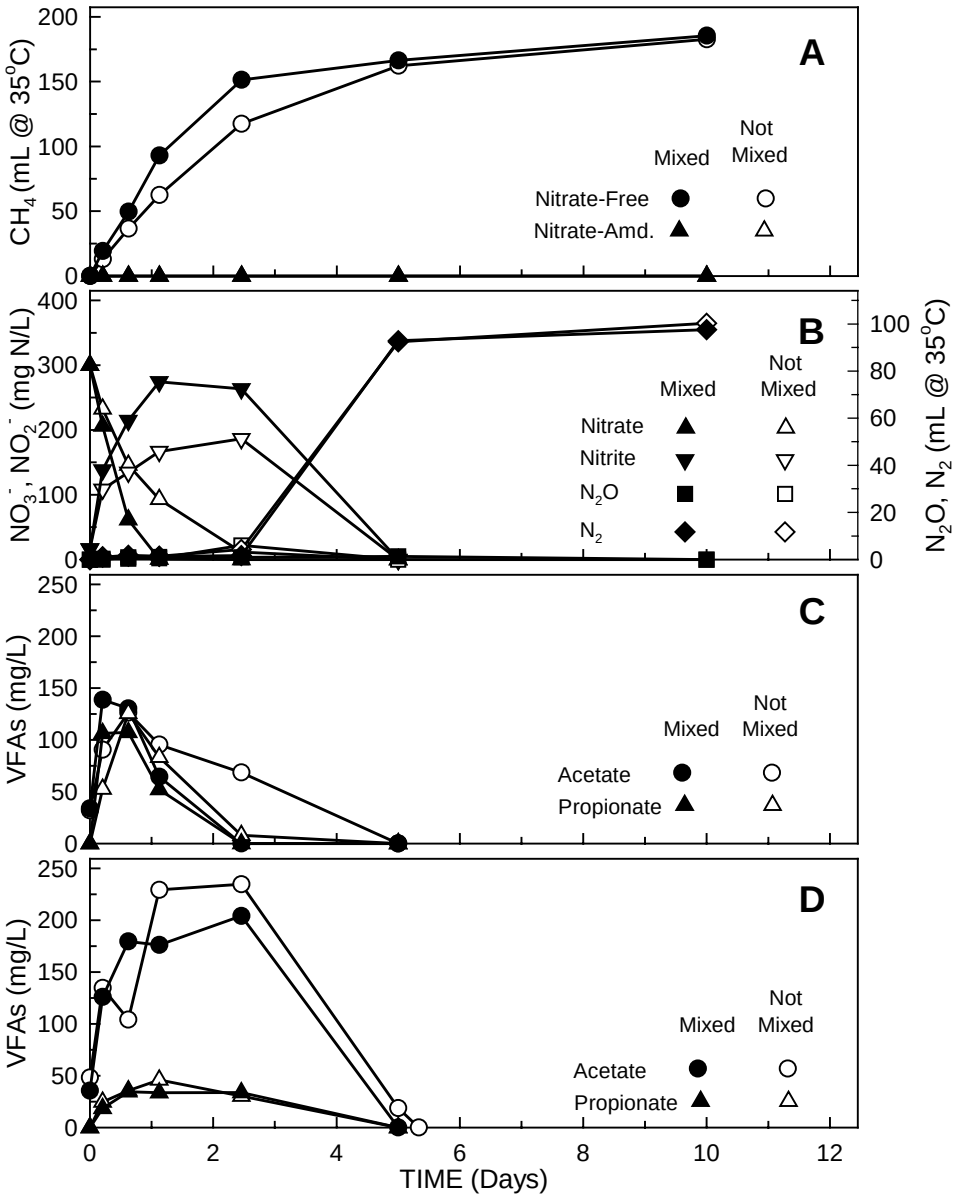

Figure 5.2. Effect of mixing on nitrate reduction and methanogenesis in a sulfide-acclimated methanogenic culture. (A) Methane production in mixed/unmixed, nitrate-free and nitrate-amended cultures, (B) Production and consumption profiles of N-oxides and N_2 in the mixed and unmixed nitrate-amended cultures, (C) VFA production and consumption profiles in mixed and unmixed, nitrate-free cultures, (D) VFA production and consumption profiles in mixed and unmixed nitrate-amended cultures. The initial nitrate concentration in the nitrate-amended cultures was 300 mg N/L. The total sulfide dose in the sulfide-amended cultures was 67 mg S/L.

observed in the absence of mixing, which is presumably due to mass transfer limitations. However, both nitrate-free cultures achieved the same extent of methane production at 10 days of incubation (Figure 5.2A). In contrast, methane production was not observed in the two nitrate-amended cultures regardless of mixing (Figure 5.2A). With mixing, nitrate-reduction occurred faster than in the unmixed culture, leading to a higher accumulation of nitrite as compared to the unmixed culture (Figure 5.2B). Similar levels of N_2O were observed in both the mixed and unmixed nitrate-amended cultures (Figure 5.2B). Although mixing affected the rates of nitrate-reduction, complete nitrogen oxide reduction occurred at the same time in both the mixed and unmixed, nitrate-amended cultures. Therefore, very similar nitrogen gas production profiles were observed in both the mixed and unmixed, nitrate-amended cultures (Figure 5.2B). Acetic, propionic and traces of isobutyric and butyric acids were observed in all cultures. Acetic and propionic acid production and consumption profiles were very similar both in the mixed and unmixed nitrate-free, control cultures (Figure 5.2C). Mixing resulted in slightly lower transient acetic and propionic acid levels in the nitrate-amended culture, compared to that in the unmixed culture (Figure 5.2D). Mass transfer limitations in the unmixed culture were the probable cause of the higher VFA levels. The results of this assay show that mixing increases the rate of nitrate reduction and thus increases the transient nitrite level. Although, methane production was not observed in neither the unmixed nor the mixed nitrate-amended cultures, it is believed that the presence of sulfide was the reason for the complete cessation of methane production resulting from the inhibition of methanogenesis brought about by the denitrification intermediates (e.g., NO and N_2O).

5.3.3. Assessment of the Effect of Sulfide on Nitrate Reduction and Methanogenesis in a Sulfide-Free Enriched Culture

The first assay, which tested the effect of sulfide on nitrate reduction and methanogenesis in a sulfide-free enriched culture lasted for 18 days. The initial pH value was 7.5 ± 0.1 (mean ± standard deviation) and the final pH values were 7.0 and 7.3; 7.0 and 7.5 for the sulfide- and nitrate-free and sulfide-free, nitrate-amended cultures; nitrate-free, sulfide-amended and sulfide- and nitrate-amended cultures, respectively. Although the final pH values in the control cultures were very similar regardless of sulfide amendment, the final pH values in the nitrate-amended cultures were significantly higher. The methane production rate and extent were similar in the sulfide-free and sulfide-amended nitrate-free cultures (Figure 5.3A). However, methane production was completely suppressed in the sulfide-, nitrate-amended culture and did not recover by the end of the incubation period. In the sulfide-free, nitrate-amended culture, methane production was suppressed at the beginning of the incubation while nitrogen oxides were still present. However, as soon as the nitrogen oxides were consumed, methanogenesis recovered (Figure 5.3A and 5.3B). In addition, although the initial rate of nitrate reduction was similar in the sulfide-free and sulfide-amended cultures for the first 6 h, cessation of nitrate reduction and accumulation of nitrite was observed in the sulfide-, nitrate-amended culture (Figure 5.3B). In contrast, nitrate reduction was not interrupted in the sulfide-free, nitrate-amended culture and complete nitrogen oxide removal was achieved in 2 d (Figure 5.3B). Subsequent to the cessation of nitrate reduction and accumulation of nitrite, nitrous oxide and nitric oxide were observed in the sulfide-, nitrate-amended culture. Because nitric oxide is a highly reactive radical (Zumft, 1993),

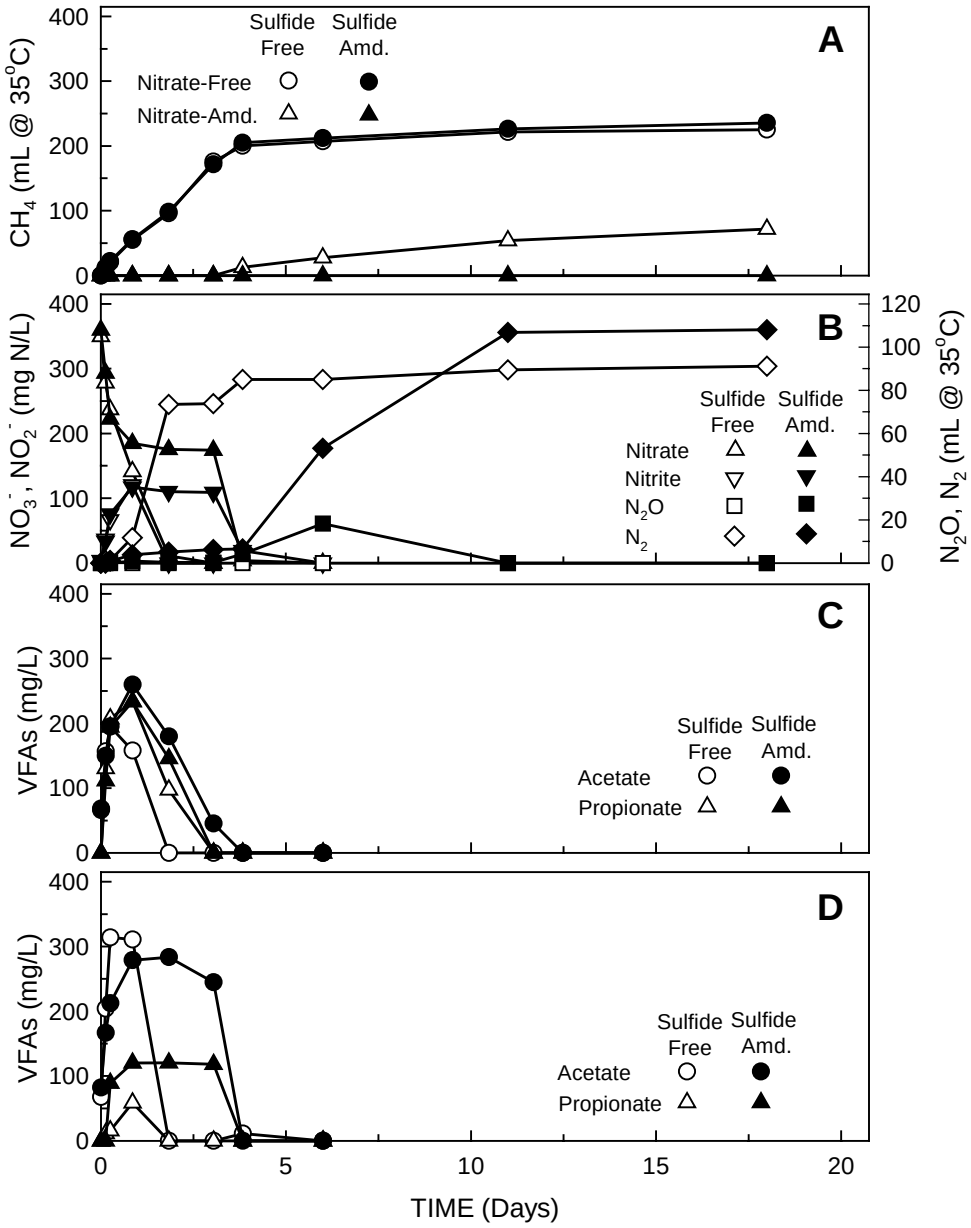

Figure 5.3. Effect of sulfide on nitrate reduction and methanogenesis in a sulfide-free enriched culture. (A) Methane production in sulfide-free and sulfide-amended nitrate-free and nitrate-amended cultures, (B) Production and consumption profiles of N-oxide species in the sulfide-free and sulfide-amended nitrate-amended cultures, (C) VFA production and consumption profiles in the nitrate-free, sulfide-free, and sulfide-amended cultures, (D) VFA production and consumption profiles in sulfide-free and sulfide-amended nitrate-amended cultures. The initial nitrate concentration in the nitrate-amended cultures was 350 mg N/L.

it was not possible to quantify low NO concentrations with the analytical technique used in this study. Therefore, the presence of NO was only qualitatively assessed based on peak appearance in gas chromatograms during nitrate reduction. As soon as the NO peak disappeared, nitrate and nitrite reduction resumed, which was followed by a slow production of nitrogen gas (Figure 5.3B).

Nitric oxide is a highly reactive radical and toxic to various bacterial and archaeal species (Zumft, 1993; Klüber and Conrad, 1998). Nitric oxide attacks to Fe groups in enzymes and therefore is especially toxic to bacteria (Culotta and Koshland, 1992). We have previously reported that an aqueous concentration of NO as low as 0.8 mg N/L in the same sulfide-free, mixed methanogenic culture inhibited acidogenesis, acetoclastic methanogenesis, as well as the reduction of the resulting N_2O to N_2 (Tugtas and Pavlostathis, 2007a). Klüber and Conrad (1998) reported that an aqueous concentration of 0.1 mg N/L nitric oxide in rice paddy field soil microcosms caused complete cessation of methanogenesis for 4 d, which subsequently recovered.

The extent of N_2 production was higher in the sulfide-, nitrate-amended culture compared to that of the sulfide-free culture. Higher N_2 production in the sulfide-amended culture could be due to autotrophic denitrifiers such as *Thiomicrospira denitrificans* and *Thiobacillus denitrificans*, which are able to use sulfide as an electron donor and produce sulfate and N_2 gas (Garcia de Lomas et al., 2005). Brunet and Garcia-Gill (1996) also reported enhanced denitrification in the presence of sulfide due to the presence of autotrophic denitrifiers.

Acetic and propionic acid production and consumption profiles were very similar in the nitrate-free, sulfide-free and sulfide-amended cultures (Figure 5.3C). Only the

acetic acid level was slightly lower in the nitrate-free, sulfide-free culture compared to the sulfide-amended culture, but the difference was statistically insignificant. However, in both sulfide-free and sulfide-amended, nitrate-amended cultures, complete acetic acid utilization was delayed in the sulfide-amended culture as compared to that in the sulfide-free culture (Figure 5.3D). Similarly, complete propionic acid utilization required a longer incubation time in the sulfide-amended culture showing a cessation concurrently with the cessation of nitrate reduction, which occurred between 20 h and 3 d of incubation (Figure 5.3D). Nitrogen gas production continued even after the complete utilization of VFAs in the sulfide-amended culture, suggesting that some other form of carbon/electron donor source was utilized after the complete utilization of VFAs. The results of this assay show that NO inhibits fermenters, methanogens, and even nitrate reducers. The observed delay of acetate and propionate oxidation in sulfide-amended cultures supports the hypothesis that sulfide was used as an alternative electron donor for the reduction of nitrate carried out by autotrophic nitrate reducers.

Ammonia concentrations were quite similar in all four cultures. However, as mentioned above, a higher N_2 production was observed in the sulfide- and nitrate-amended culture. About 84% of the expected N_2 was produced in the sulfide-free culture, whereas approximately 97% of the expected N_2 was produced in the sulfide-amended culture, suggesting that nitrate reduction did not occur via DNRA in both the sulfide-free and the sulfide-amended cultures. The results of this assay show that in the presence of sulfide, accumulation of denitrification intermediates such as NO and N_2O takes place, which irreversibly inhibit methanogens and result in the complete cessation of methane production. Similar results were obtained by Brunet and Garcia-Gill (1996), who found

that the presence of sulfide (32 mg S/L) in a stratified lake caused incomplete denitrification mainly to NO and ammonia through partial inhibition of NO and N_2O reductases. Senga et al. (2006) reported that H_2S caused accumulation of nitrite and N_2O and resulted in a shift from denitrification to DNRA. In addition, Cardoso et al. (2006) also reported that sulfide concentrations higher than 2.5 mM (80 mg S/L) resulted in inhibition of nitrate reduction in a chemolithoautotrophic enrichment culture.

The second assay conducted with the sulfide-free enriched methanogenic culture tested the effect of varying concentrations of sulfide on nitrate reduction and methanogenesis and lasted for 126 days. The initial/final pH values for the nitrate-free and 0 (control), 10, 20, 40, 80, and 100 mg S/L sulfide-amended cultures were 7.2/7.0, 7.3/7.0, 7.5/7.1, 7.5/7.1, 7.7/7.2, and 7.8/7.3, respectively. The initial/final pH values for the 120 mg N/L nitrate-amended cultures were 7.2/7.0, 7.3/7.1, 7.4/7.1, 7.6/7.3, 7.7/7.2, and 7.7/7.4 for the 0, 10, 20, 40, 80, and 100 mg S/ L and sulfide-amended cultures, respectively. Methane production rates were similar in all nitrate-free, sulfide-amended cultures (Figure 5.4A). However, the extent of methane production was slightly lower in the sulfide-amended cultures compared to that of the control (i.e., sulfide-free) culture (Figure 5.4A). Methane production completely ceased in all nitrate- and sulfide-amended cultures, and recovered only in the 10 mg S/L sulfide-amended cultures (Figure 5.4B). Methane production only partially recovered in the 10 mg S/L sulfide- and nitrate-amended culture and occurred approximately 40 days after the complete utilization of all nitrogen oxides.

Although the nitrate reduction rate was the same in all sulfide-amended cultures regardless of the initial sulfide concentration, nitrite reduction became slower as the

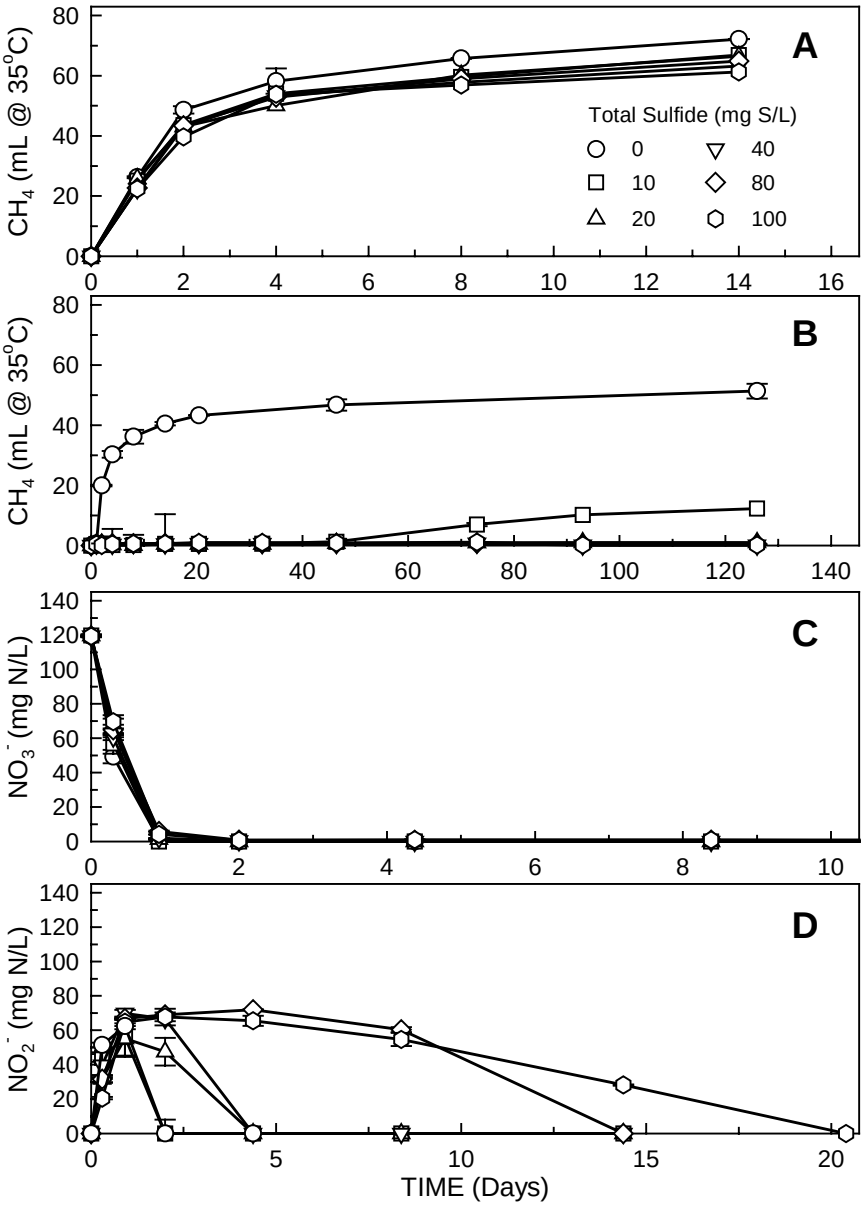

Figure 5.4. Effect of sulfide concentration on nitrate reduction and methanogenesis. (A) Methane production in the nitrate-free, sulfide-amended, cultures, (B) Methane production in the sulfide- and nitrate-amended cultures, (C) Nitrate reduction in the nitrate- and sulfide-amended cultures, (D) Nitrite production and consumption in the nitrate- and sulfide-amended cultures. The initial nitrate concentration in the nitrate-amended cultures was 120 mg N/L. Error bars represent mean values ± one standard deviation (n = 3).

initial sulfide concentration increased (Figure 5.4C and D). As the initial sulfide concentration increased from 0 to 100 mg S/L, a higher nitrite, nitric oxide, and nitrous oxide accumulation was observed (Figure 5.4D and Fig. 5.5A). Nitrite and nitrous oxide accumulated in the 80 and 100 mg S/L sulfide-amended cultures, for 14 and 20 days, respectively (Figures 5.4D, 5.5A, and Table 5.1). Although the rate of nitrate reduction was approximately the same in all nitrate- and sulfide-amended cultures, due to the accumulation of intermediate nitrogen oxides, the nitrogen gas production rates decreased as the initial sulfide concentration increased (Table 5.1). Although the rate of nitrogen gas production was the lowest in the 100 mg S/L sulfide-amended culture, the extent of nitrogen production in this culture was about the same as in the sulfide-free, nitrate-amended (control) culture. Within experimental error, all sulfide-amended cultures produced about the same volume of nitrogen gas, which suggests that DNRA did not occur in any of the sulfide-amended cultures (Figure 5.5B). Acetic and propionic acids along with low levels of butyric acid were observed in the control (i.e., sulfide-free) culture (Figure 5.6A). In all the sulfide-amended, nitrate-free cultures the VFA profiles were similar to those in the control culture (Figure 5.7). VFAs accumulated and were not consumed in the sulfide- and nitrate-amended cultures for an incubation period of 126 days (Figure 5.6B and C). VFA data are shown only for the 10 and 100 mg S/L sulfide- and nitrate-amended cultures (Fig. 5.6B and C). Despite the abundance of carbon sources after the complete conversion of nitrate to nitrogen gas, methane production did not recover in any of the sulfide- and nitrate-amended cultures except for the 10 mg S/L sulfide-amended culture in which case methane production recovered only partially (Fig. 5.4B). The results of COD balance calculations are reported in Table 5.1. The following

Table 5.1. Methane production, nitrate reduction and COD utilization in mixed methanogenic cultures amended with nitrate and sulfide (six levels).

Culture Series/ Sulfide (mg S/L)	Methane Production	Nitrate Reduction			COD Processed (%)			
	Time (d)[b]	Time (d)[e]	Nitrogen gas production rate (mL/d)[f]	r^2	CH_4	VFAs	Nitrate reduction[g]	Total[h]
0 (Control)[a]	0	0	0	0	100	0	0	100
0	1	2	8.5 ± 0.2	0.99	67.2	0	18.8	86.0
10	46	4	7.4 ± 0.2	0.99	17.1	63.9	18.8	99.8
20	73[c]	4	3.5 ± 0.1	0.99	1.4	69.9	18.8	90.1
40	ND[d]	8	3.3 ± 0.1	0.99	0	67.1	18.8	85.9
80	ND	14	1.0 ± 0.2	0.96	0	53.7	18.8	72.5
100	ND	20	0.7 ± 0.1	0.97	0	42.6	18.8	61.4

[a] Sulfide- and nitrate-free culture; all other cultures were amended with sulfide and 120 mg N/L nitrate.
[b] Incubation time which the methane production was first observed.
[c] Traces
[d] ND, not detected.
[e] Time required for the complete N-oxide reduction to N_2.
[f] Mean ± standard deviation values estimated by linear regression of initially produced nitrogen gas volume ($n \geq 3$; $R^2 > 0.96$).
[g] Fraction (%) of COD utilized for the complete conversion of nitrate to N_2 neglecting microbial growth (calculated).
[h] Normalized to the total COD utilized for methane production in the control (i.e., sulfide- and nitrate-free) culture.

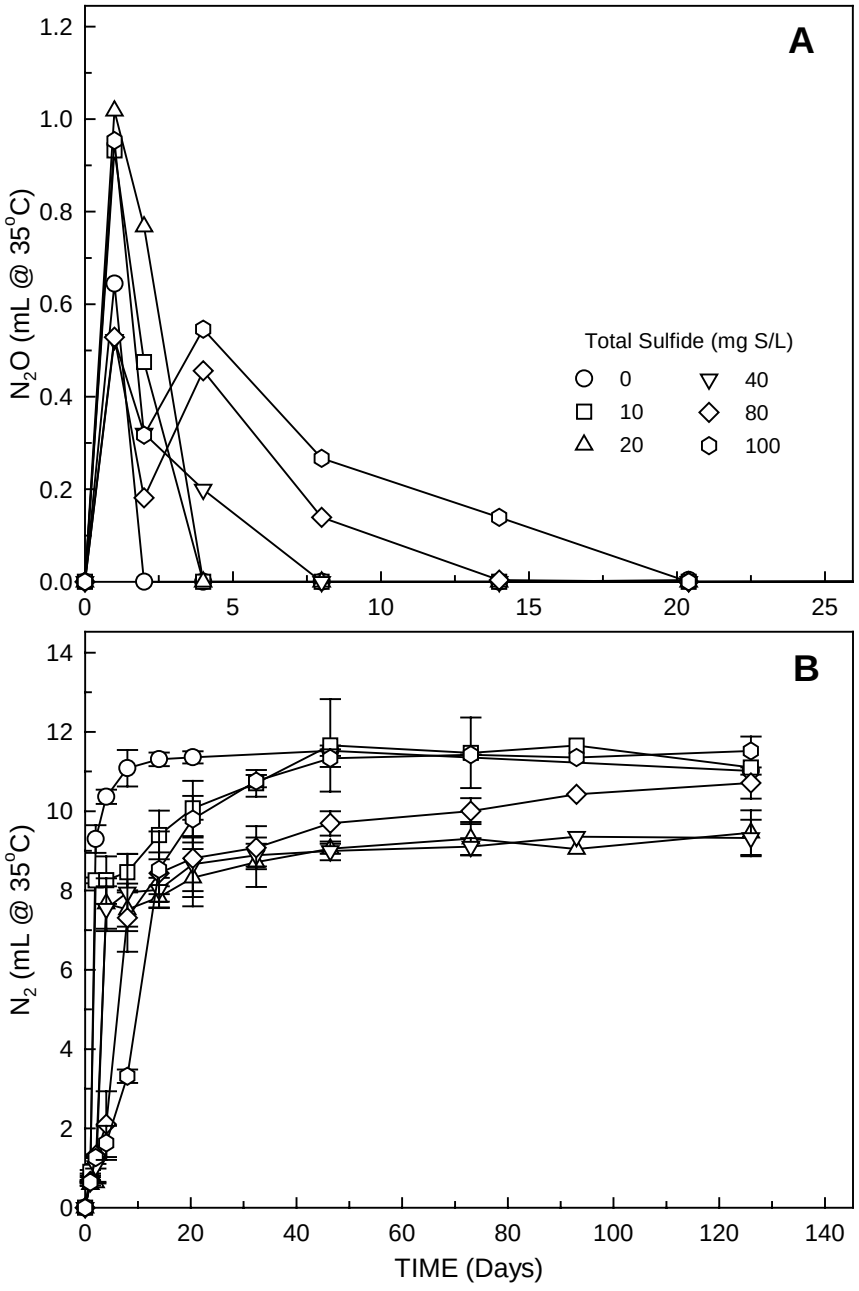

Figure 5.5. Effect of sulfide concentration on nitrate reduction and methanogenesis. (A) Nitrous oxide production and consumption in the sulfide- and nitrate-amended cultures, (B) N_2 gas production in the sulfide- and nitrate-amended cultures. The initial nitrate concentration in the nitrate-amended cultures was 120 mg N/L. Error bars represent mean values ± one standard deviation (n = 3).

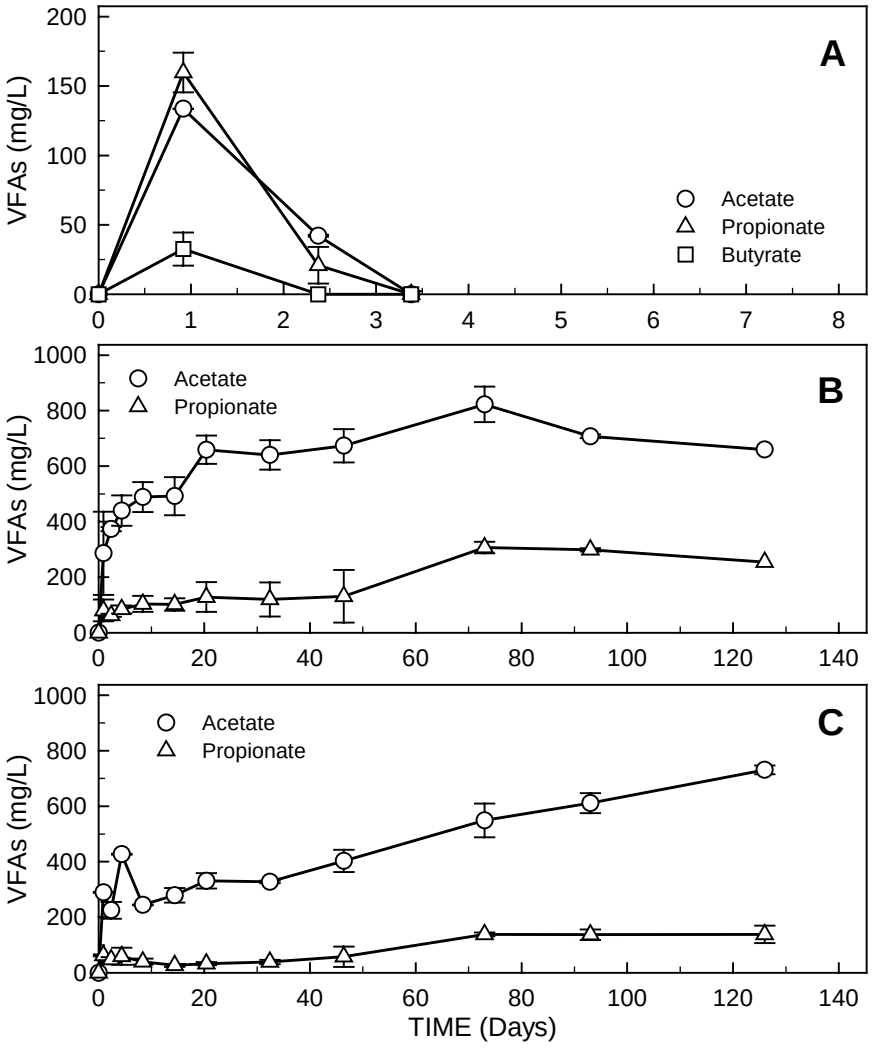

Figure 5.6. Effect of sulfide concentration on nitrate reduction and methanogenesis. VFA production and consumption profiles in nitrate-amended cultures at initial total sulfide concentration of (A) 0 mg S/L, (B) 10 mg S/L, (C) 100 mg S/L. The initial nitrate concentration in the nitrate-amended cultures was 120 mg N/L. Error bars represent mean values ± one standard deviation (n = 3).

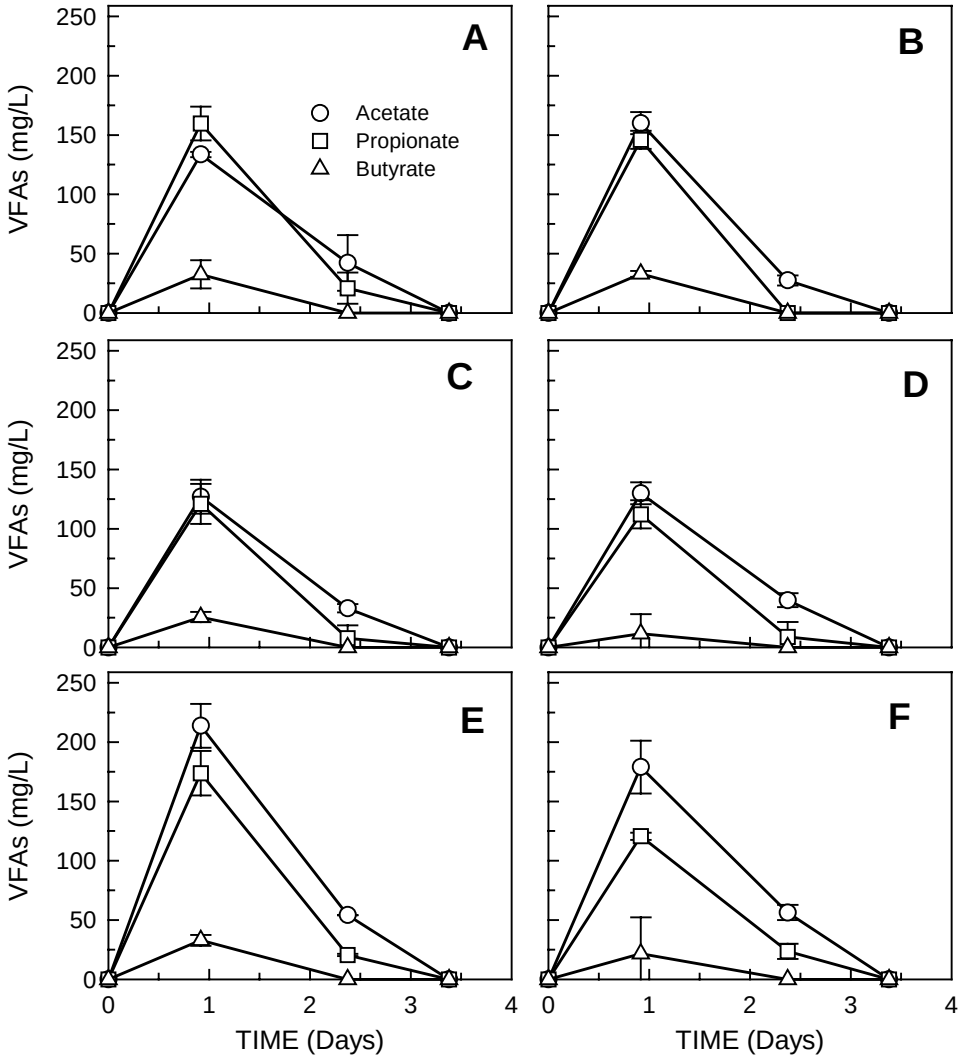

Figure 5.7. Effect of sulfide concentration on methanogenesis. VFA production and consumption profiles in nitrate-free cultures at initial total sulfide concentration of (A) 0 mg S/L, (B) 10 mg S/L, (C) 20 mg S/L, (D) 40 mg S/L, (E) 80 mg S/L, (F) 100 mg S/L. Error bars represent mean values ± one standard deviation (n = 3).

COD fractions were considered in these calculations: COD processed for methane production, COD of the unutilized VFAs, and the theoretical amount of COD required for the complete N-oxide reduction (2.857 mg COD/mg NO_3^--N). As shown in Table 5.1, total processed COD in the 40, 80, and 100 mg S/L sulfide-amended cultures was lower compared to that in the control, 10, and 20 mg S/L sulfide-amended cultures, suggesting that at higher sulfide concentrations, fermentation was inhibited. Inhibition of fermentation may have resulted in the accumulation of unprocessed COD, and/or unaccounted fermentation products resulting in less than 100% COD balance closure (Table 5.1).

In order to understand the fate of sulfur species in the sulfide-amended cultures, the MINEQL+ version 4.5 chemical equilibrium software was used (Schecher and McAvoy, 1992). The calculations were carried out under the conditions of the sulfide-free methanogenic culture used in the present study: pH 6.9, temperature 35^{o}C, ionic strength 0.104 M, and alkalinity 3.9 g/L as $CaCO_3$. In addition, all the calculations were conducted under closed system conditions and total carbonate levels were calculated using pH and alkalinity values. The initial ORP value of each culture was measured and converted to E_H (in V). Then, the redox potential (pε) values were calculated using the Nernst equation (pε = 16.3 E_H at 35^{o}C). The initial E_H [pε] values for the nitrate-free and 0 (control), 10, 20, 40, 80, and 100 mg S/L sulfide-amended cultures were -103.9 ± 12.0 [-1.7], -121.0 ± 5.9 [-2.0], -113.1 ± 17.2 [-1.9], -107.9 ± 16.0 [-1.8], -145.1 ± 28.7 [-2.4] and, -143.1 ± 6.0 [-2.3] mV (mean ± standard deviation), respectively. The initial E_H [pε] values for the 120 mg N/L nitrate-amended cultures were 22.0 ± 7.7 [0.4], -18.8 ± 9.0 [-0.3], -16.9 ± 34.4 [-0.3], -11.1 ± 5.2 [-0.2], -114.3 ± 12.2 [-1.9], and –118.9 ± 15.4 [-1.9]

(mean ± standard deviation) for the 0, 10, 20, 40, 80, and 100 mg S/L and sulfide-amended cultures, respectively. Free- and total-sulfides were measured and sulfide speciation was estimated using MINEQL+. The measured soluble and total sulfide along with the calculated aqueous and precipitated sulfide concentrations are shown in Table 5.2. According to MINEQL+ speciation modeling, HS^-, H_2S (aq), $Cu(HS)_3^-$ (aq), $Fe(HS)_2$, CoS, sphalerite (ZnS), and mackinawite ($(FeNi)_9S_8$) were found to be the most dominant sulfur species in the culture used in this study. The presence of nitrate did not change the total amount of the sulfur species formed. However, the type of sulfur speciation changed in the presence of nitrate (Table 5.3). Measured soluble- and total-sulfide was lower than the calculated in the cultures amended with 120 mg N/L nitrate (Table 5.2). However, higher sulfide recoveries were obtained in the nitrate-free, sulfide-amended cultures. The measured soluble sulfide varied from 15 to 77% of the total sulfide in the nitrate-free, 10 to 100 mg S/L sulfide-amended cultures. However, in the nitrate-amended cultures, soluble sulfide was detected only in the 80 and 100 mg S/L sulfide-amended cultures and represented 43 and 63% of the total sulfide, respectively. Decreased levels of measured sulfide in the presence of nitrate could be an indication of conversion of sulfide to other sulfur species (e.g., sulfate). In addition, during the incubation period, yellow precipitates were observed in the 40, 80, and 100 mg S/L sulfide- and nitrate-amended cultures, which is an indication of elemental sulfur formation due to partial oxidation of sulfide in the presence of nitrate. Reyes-Avila et al. (2004) suggested that under denitrifying conditions, sulfide is partially oxidized to elemental sulfur with a concomitant reduction of nitrate to nitrite and then the elemental sulfur is oxidized to sulfate concomitant to reduction of nitrite to N_2. As discussed above, autoclaved control assays showed that

Table 5.2. Measured soluble/total sulfide levels and calculated aqueous and precipitated sulfide levels in sulfide-amended, nitrate-free and nitrate-amended cultures.

Initial Sulfide (mg S/L)	Measured Sulfide (mg S/L)[c]		Total Sulfide Recovered (%)[c]	Calculated Sulfide (M) [mg/L][c]	
	Soluble	Total		Aqueous	Precipitated
Nitrate-Free Cultures					
0	ND[b]	ND	-	-	-
10	1.5 ± 0.2	8.2 ± 0.2	82 ± 2	9.25×10^{-5} [3.0]	2.20×10^{-4} [7.0]
20	2.4 ± 0.3	17.6 ± 2.0	88 ± 10	6.4×10^{-5} [2.1]	5.61×10^{-4} [17.9]
40	13.6 ± 0.7	33.2 ± 0.6	83 ± 1.5	6.14×10^{-4} [19.6]	6.36×10^{-4} [20.4]
80	46.4 ± 2.5	68.8 ± 3.1	86 ± 3.9	1.88×10^{-3} [60.3]	6.17×10^{-4} [19.7]
100	77.0 ± 5.2	100.0 ± 3.6	100 ± 3.6	2.52×10^{-3} [80.5]	6.10×10^{-4} [19.5]
Nitrate-Amended Cultures[a]					
0	ND	ND	-	-	-
10	0	0	0	2.05×10^{-5} [0.6]	2.92×10^{-4} [9.4]
20	0	0	0	6.5×10^{-5} [2.1]	5.60×10^{-4} [17.9]
40	0	4.4 ± 0.3	11 ± 0.8	6.20×10^{-4} [19.8]	6.30×10^{-4} [20.2]
80	34.4 ± 0.9	53.6 ± 2.2	67 ± 2.8	1.89×10^{-3} [60.4]	6.14×10^{-4} [19.6]
100	63 ± 1.9	87 ± 7.9	87 ± 7.9	2.52×10^{-3} [80.6]	6.03×10^{-4} [19.4]

[a] Amended with 120 mg N/L nitrate at the beginning of the incubation.
[b] ND, not detected.
[c] Mean ± standard deviation ($n = 3$).
[d] Calculated using MINEQL+, version 4.5 chemical equilibrium software; bracketed numbers are expressed in terms of mg S/L.

Table 5.3. Calculated major sulfur species in sulfide-amended, nitrate-free and nitrate-amended cultures[a].

Initial Sulfide (mg S/L)	Sulfur Species (M)						
	H_2S (aq)	HS^-	$Cu(HS)_3^-$ (aq)	$Fe(HS)_2$ (aq)	CoS	ZnS	$(FeNi)_9S_8$
Nitrate-Free Cultures							
10	9.6×10^{-5}	1.3×10^{-4}	0	3.7×10^{-8}	8.4×10^{-6}	3.7×10^{-6}	8.9×10^{-6}
20	9.7×10^{-5}	1.3×10^{-4}	0	3.9×10^{-8}	8.4×10^{-6}	3.7×10^{-6}	4.8×10^{-5}
40	3.1×10^{-4}	4.1×10^{-4}	0	4.0×10^{-7}	8.4×10^{-6}	3.7×10^{-6}	6.1×10^{-5}
80	8.5×10^{-4}	1.1×10^{-3}	0	2.9×10^{-6}	8.4×10^{-6}	3.6×10^{-6}	5.9×10^{-5}
100	1.1×10^{-3}	1.5×10^{-3}	0	5.1×10^{-6}	8.4×10^{-6}	3.6×10^{-6}	5.8×10^{-5}
Nitrate-Amended Cultures							
10	9.5×10^{-5}	1.2×10^{-4}	2.7×10^{-6}	3.7×10^{-8}	8.4×10^{-6}	3.7×10^{-6}	9.3×10^{-6}
20	9.6×10^{-5}	1.3×10^{-4}	2.7×10^{-6}	3.8×10^{-8}	8.4×10^{-6}	3.7×10^{-6}	4.9×10^{-5}
40	3.2×10^{-4}	4.2×10^{-4}	9.0×10^{-6}	4.1×10^{-7}	8.4×10^{-6}	3.6×10^{-6}	6.1×10^{-5}
80	8.7×10^{-4}	1.1×10^{-3}	2.4×10^{-5}	2.9×10^{-6}	8.4×10^{-6}	3.6×10^{-6}	5.9×10^{-5}
100	1.2×10^{-3}	1.5×10^{-3}	3.2×10^{-5}	5.1×10^{-6}	8.4×10^{-6}	3.6×10^{-6}	5.8×10^{-5}

[a] Based on MINEQL+ calculations

nitrate reduction in the cultures used in the present study was entirely due to biological activity. Therefore, the decrease in the sulfide concentration in the nitrate-amended cultures could be due to conversion of sulfide to sulfate in these cultures through autotrophic nitrate reduction. An increase in the sulfate concentration was observed in the 40, 80, and 100 mg S/L sulfide-amended cultures measured at the end of the incubation. It should be noted that the culture media used in the present study contain sulfate as part of the trace metal solution (0.02 mg SO_4^{2-}-S/L) and the media contain sulfide (0.16 mg S/L). Thus, the media contribution in terms of total sulfide concentration is less than 0.2 mg S^{2-}/L. Production of sulfate at the end of the incubation agrees with autotrophic nitrate reduction leading to conversion of sulfide to sulfate as previously reported (Krishnakumar and Manilal, 1999). MINEQL+ calculations indicated that, in the presence of nitrate, the sulfur speciation only slightly changed compared to that of the nitrate-free cultures (Table 5.3). MINEQL+ calculations resulted in a lower free-sulfide concentration in the presence of 120 mg N/L nitrate compared to that of the nitrate-free culture in the 10 mg S/L amended culture (Table 5.2). Sulfur speciation in the 10, 20, 40, 80, and 100 mg S/L amended cultures with and without nitrate were calculated using MINEQL+. The results of these calculations also showed that the addition of nitrate did not result in significant changes in the sulfur speciation (Table 5.3). However, nitrate resulted in the formation of $Cu(HS)_3^-$ which was presumably due to the increase in the redox value as a result of nitrate addition (Table 5.3). This result also indicates that nitrate reduction did not occur through chemical reaction(s), but was solely due to microbial reduction. The nitrate reduction rate was the same in all sulfide-amended cultures (Figure 5.4C), suggesting that nitrate reduction was not inhibited by sulfide.

However, the reduction rate of nitrite and nitrous oxide decreased as the initial sulfide concentration increased, suggesting that both the nitrite and nitrous oxide reduction were inhibited by sulfide (Figures 5.4D and 5.5A). Although both the nitric and nitrous oxide reduction are inhibited in the presence of sulfide, it is known that nitrous oxide reduction is more susceptible to sulfide (Delwiche, 1981). The results of the present study show that in the presence of nitrate and sulfide, free sulfide species exert an inhibitory effect on nitrite and nitrous oxide reduction resulting in long-term accumulation of these species under the conditions of the cultures used in the present study. Long-term accumulation of denitrification intermediates causes inhibition of fermentation and methanogenesis, leading to accumulation of VFAs and complete cessation of methane production. The inhibitory effect of sulfide on both the denitrifying and methanogenic microorganisms may be the result of deficiency in essential metal cofactor(s) resulting from metal sulfide precipitation. Sulfide inhibition of N_2O reduction to N_2 was reversed by the addition of 60 μM copper in denitrifying sludge cultures (Manconi et al., 2006).

5.3.4. Assessment of the Effect of Nitrate on Sulfide-Amended and Sulfide-Acclimated Cultures

This assay, which tested the effect of nitrate and sulfide on sulfide-amended and sulfide-acclimated cultures, lasted for 35 days. The initial pH values in the sulfide-amended and sulfide-acclimated cultures were 7.6 ± 0.1 and 7.3 ± 0.1(mean ± standard deviation), respectively. The final pH values in the sulfide-amended control (i.e., nitrate-free) and nitrate-amended cultures were 7.0 and 7.2, respectively. Likewise, the final pH values in the sulfide-acclimated control (i.e., nitrate-free) and nitrate-amended cultures were 7.1

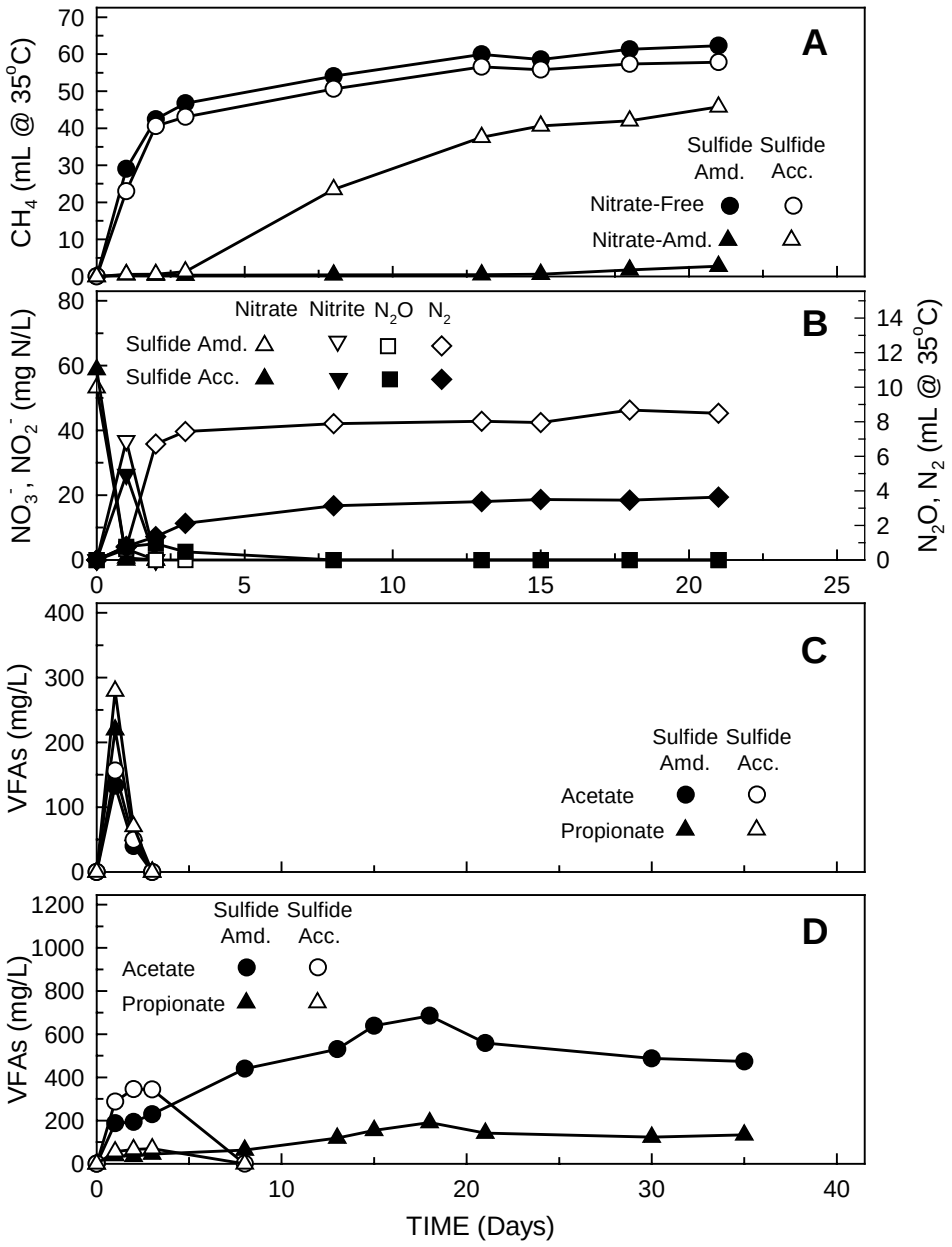

Figure 5.8. Effect of nitrate on sulfide-amended and sulfide-acclimated cultures. (A) Methane production in sulfide-amended and sulfide-acclimated nitrate-free and nitrate-amended cultures, (B) Production and consumption profiles of N-oxide species and N_2 in the sulfide-amended and sulfide-acclimated nitrate-amended cultures, (C) VFA production and consumption profiles in the nitrate-free, sulfide-amended and sulfide-acclimated cultures, (D) VFA production and consumption profiles in sulfide-amended and sulfide acclimated nitrate-amended cultures. The initial nitrate concentration in the nitrate-amended cultures was 75 mg N/L.

and 7.4, respectively. The methane production rate and extent were similar in the sulfide-amended and sulfide-acclimated control cultures, in agreement with previous observations (see above) (Figure 5.8A). Similar to previous observations, for all practical purposes, methane production did not recover in the sulfide- and nitrate-amended culture and only traces of methane were detected towards the end of the incubation period (Figure 5.8A). In contrast, recovery of methane production was observed in the sulfide-acclimated culture even though the level of total sulfide in this culture was similar to that of the sulfide-amended culture. Nitrate reduction and intermediate N-oxides production and consumption profiles were similar in both the sulfide-amended and sulfide-acclimated cultures (Figure 5.8B). Peaks representative of low NO concentrations were observed in gas chromatograms, but as mentioned above, NO concentrations could not be accurately quantified. The nitrogen gas produced in the sulfide-acclimated culture was only 43% as compared to that in the sulfide-amended culture. In addition, ammonia production, which was observed in the sulfide-acclimated culture at the end of the incubation period was in excess of that found in the control (i.e., nitrate-free) culture. However, the increased ammonia production could not be accurately determined due to the peptone-derived ammonia production. As discussed above (see Section 5.2.1), peptone used in this study contains 8% total nitrogen, which, based on the peptone concentration used in this assay and assuming complete peptone degradation, may result in the production of 38 mg NH_4^+-N/L.

Acetic and propionic acid production and consumption profiles were similar in the nitrate-free, sulfide-amended and sulfide-acclimated cultures (Figure 5.8C). Acetic and propionic acid production and consumption occurred within 8 days in the sulfide-

acclimated, nitrate-amended culture (Figure 5.8D). However, long-term accumulation of acetic and propionic acids was observed in the sulfide- and nitrate-amended culture and methane production was not observed (Figure 5.8D).

Although the inoculation and feeding procedure were the same for both the sulfide-free and sulfide-acclimated cultures, amending the sulfide-free enriched culture with the same amount of sulfide as that of the sulfide-acclimated culture resulted in complete cessation of methanogenesis, accumulation of acetic and propionic acids, and denitrification took place as the dominant pathway of nitrate reduction. In contrast, although the nitrate and nitrite reduction profiles in the sulfide-acclimated culture were the same as in the sulfide-amended culture, as discussed above, nitrate was converted to both nitrogen gas and ammonia in the sulfide-acclimated culture through denitrification and DNRA pathways, respectively. The switch to DNRA prevented the accumulation of toxic denitrification intermediates converting nitrite to ammonia, and methanogenesis recovered after the complete utilization of all nitrogen oxides. These results suggest that during the reduction of nitrate, sulfide leads to accumulation of NO, which in turn irreversibly inhibits methanogenesis in sulfide-free enriched cultures when amended with sulfide. Predominance of DNRA in cultures acclimated to sulfide prevents inhibition of methanogenesis. However, in the latter case, ammonia is produced as opposed to nitrogen gas, thus reducing the effectiveness of such a system to achieve a high degree of nitrogen removal.

5.3.4. Assessment of the Effect of the COD/N Value on Nitrate Reduction in a Sulfide-Acclimated Culture

This assay, which tested the effect of COD/N value on nitrate reduction, lasted for 7 days. The initial/final pH values for the control (i.e., nitrate-free), 10, 20, and 60 mg COD/mg NO_3^--N cultures were 7.3/7.1, 7.3/7.2, 7.2/7.1, and 7.4/7.2, respectively. Regardless of the COD/N value, complete nitrate and nitrite reduction occurred within a day. However, higher accumulation of N_2O was observed at lower COD/N values. Therefore, the lowest and the highest N_2O accumulations were observed at COD/N values of 60 and 10, respectively (Figure 5.9A). In addition, the nitrogen gas production changed as a function of COD/N value, with the highest value observed at a COD/N value of 10 and the lowest at a COD/N value of 60 (Figure 5.9A and Table 5.4). Ammonia production was the highest in the culture with an initial COD/N value of 60 (Table 5.4). Therefore, as the COD/N ratio increased from 10 to 60, lower N_2O and N_2 production and higher ammonia production was observed suggesting predominance of DNRA at higher COD/N values. Similar to our results, Akunna et al. (1992) reported that DNRA was the dominant nitrate reduction pathway at COD/N values greater than 53 in cultures fed with glucose as the sole carbon source. Nitrogen balance calculations based on initial and final nitrogen species also suggest that the decreased N_2 production corresponds to increased ammonia production (Table 5.4). The fraction of nitrate that was reduced through denitrification in the cultures with an initial COD/N value of 10, 20, and 60, was 81, 30, and 7%, respectively. The balance of the nitrate amendment was converted to ammonia via DNRA (Table 5.4). Because the feed solution for this assay consisted of only dextrin, it did not provide any additional nitrogen to the media. In addition, the initial and final

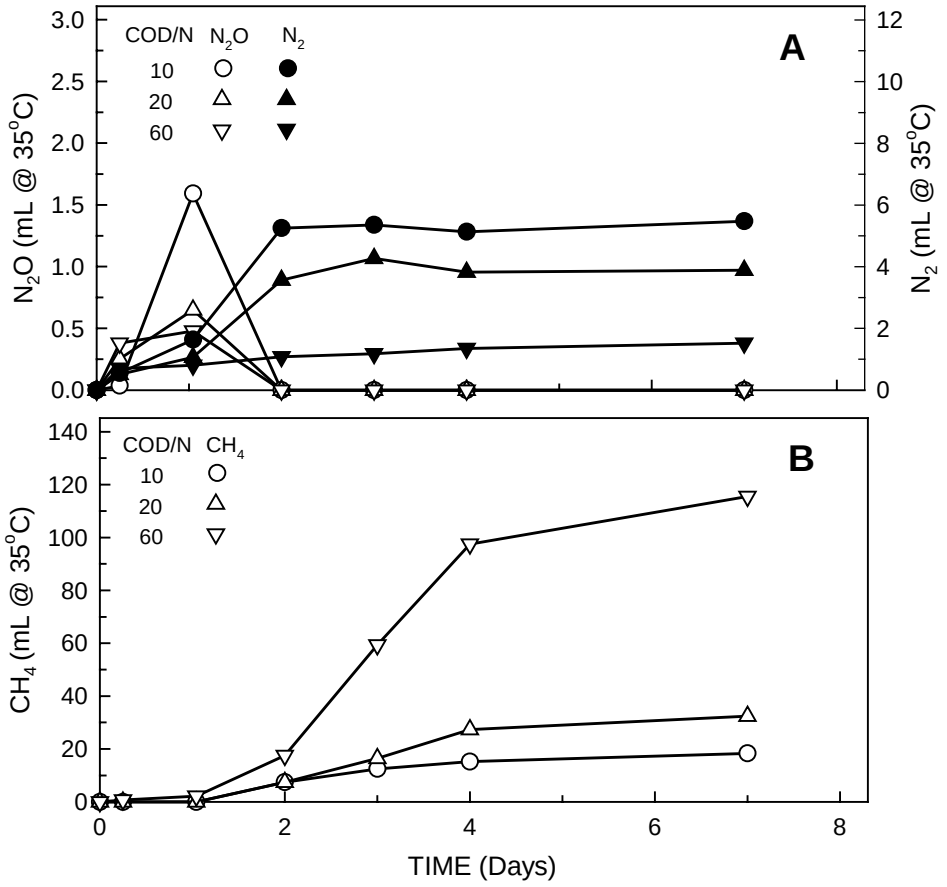

Figure 5.9. Effect of COD/N value on nitrate reduction in a sulfide-acclimated culture. (A) Nitrous oxide and N_2 gas profiles and (B) Methane production.

Table 5.4. Nitrogen balance at different COD/N values in a sulfide-acclimated, nitrate- and sulfide-amended culture.

Nitrogen Speciation	COD/N Value			
	-[a]	10	20	60
Initial Nitrogen Mass (mg N)				
Ammonia	18	17.9	18	18
Nitrate	0	8.6	8.6	8.6
Total	18	26.5	26.6	26.6
Final Nitrogen Mass (mg N)				
Ammonia	17.4	18.9	21.7	23.4
Nitrogen gas	0	4.2	1.6	0.4
Total	17.4	25.7	27.3	27.5
Nitrate Reduction Pathway (%)				
DNRA	-	19	70	93
Denitrification	-	81	30	7

[a] Nitrate-free culture; all remaining cultures were amended with 50 mg NO_3^--N/L and 67 mg S/L.

biomass concentrations were reasonably similar, therefore, the nitrogen that may be released to the media through biomass decay was not considered in the nitrogen balance calculations. Methane production was observed in the cultures for all COD/N values and the extent of methane production reached expected levels in all cultures. The difference in methane production reflects the different amount of initial COD supplied to each culture. The results of this study show that in the sulfide-acclimated culture, nitrate reduction occurred via both denitrification and DNRA at all COD/N levels, and the fraction of nitrate reduced via DNRA increased as the COD/N value increased.

5.4. Summary

The effect of sulfide on nitrate reduction and methanogenesis was investigated in two mixed, mesophilic (35^{o}C) methanogenic cultures: sulfide-free and sulfide-acclimated. Autoclaved controls (deionized water, sulfide-free and sulfide-amended media and cultures) showed that nitrate reduction in active methanogenic cultures was biologically mediated. The effect of sulfide was first tested on the sulfide-free, enriched culture by amending this culture with 67 mg S/L sulfide and 350 mg N/L nitrate. In the presence of sulfide and nitrate, methane production completely ceased and did not recover due to accumulation of denitrification intermediates. A second assay was conducted in which the sulfide-free culture was amended with 67 mg S/L sulfide and both the sulfide-free and sulfide-acclimated cultures were amended with 75 mg N/L nitrate. Methane production did not recover in the sulfide-amended culture but it did recover in the sulfide-acclimated culture. Conversion of nitrite to ammonia via DNRA prevented the accumulation of denitrification intermediates and thus methanogenesis

recovered in the sulfide-acclimated culture. The effect of sulfide concentration on nitrate reduction and methanogenesis was investigated with the sulfide-free methanogenic culture at a concentration range of 10-100 mg S/L. Denitrification was the predominant pathway at all sulfide concentration levels tested and methanogenesis did not recover in these cultures, except in the 10 mg S/L sulfide-amended culture. Denitrification was assessed with the sulfide-acclimated methanogenic culture at COD/N values of 10, 20, and 60. As the COD/N value increased, the fraction of nitrate reduced through DNRA also increased. Chemical equilibrium calculations based on the MINEQL+ software assisted in the interpretation of the effect of sulfide on the nitrate reduction and methanogenesis.

CHAPTER 6

UTILIZATION OF DIFFERENT ELECTRON DONORS FOR NITRATE REDUCTION AND METHANOGENESIS: PATHWAY AND KINETICS

6.1. Introduction

In contrast to available information regarding the effect of nitrate and denitrification intermediates on mixed fermentative and methanogenic systems (Klüber and Conrad, 1998a; Klüber and Conrad, 1998b; van Bodegom and Stams, 1999; Clarens et al., 1998; Roy and Conrad, 1999; Chidthaisong and Conrad, 2000; Tugtas and Pavlostathis, 2007a), little is known about the nitrate reduction kinetics in a mixed methanogenic culture with respect to different electron donors. The effects of denitrification intermediates and sulfide on nitrate reduction and methanogenesis in sulfide-free and sulfide-acclimated mixed methanogenic cultures have been described in Chapters 4 and 5, respectively. Substrate competition between nitrate reducers (denitrification and dissimilatory nitrate reduction to ammonia), methanogens, and other fermentative species in combined treatment systems is not well understood. A systematic assessment of the preferential utilization of different electron donors and the kinetics of nitrate reduction in the presence of different electron donors in mixed, overall methanogenic systems is lacking.

The objectives of the research reported here were to: (a) investigate the utilization of different electron donors by denitrifiers; (b) determine the predominant pathway of nitrate reduction as a function of carbon/electron donor source as well as the COD/N ratio; and (c) investigate the kinetics of nitrate reduction with respect to different electron donors in a mixed, sulfide-acclimated methanogenic culture.

6.2. Materials and Methods

6.2.1 Electron Donor Utilization Assay

A batch assay was performed to test the effect of different electron donors on the mixed methanogenic culture in the presence of nitrate using 160-mL serum bottles (110 mL liquid volume) sealed with rubber stoppers and aluminum crimps and pre-flushed with helium gas. An aliquot of 70 mL of the sulfide-acclimated methanogenic culture and 20 mL culture media were anaerobically transferred to each serum bottle. Four different substrates were used in this assay: a mixture of dextrin and peptone (D/P), propionate, acetate, and H_2/CO_2. Each electron donor was added to serum bottles resulting in an initial chemical oxygen demand (COD) of 500 mg/L. In order to provide a total of 500 mg COD/L with a H_2/CO_2 mixture (80% to 20% v/v, respectively), 332 mL of the H_2/CO_2 mixture at 35^{o}C was added to the bottle headspace. For each substrate, one of the serum bottles was nitrate-free and the other one was amended with 50 mg N/L nitrate using a $NaNO_3$ stock solution. The COD/N value of 10 was selected in order to prevent the prevalence of DNRA in this experiment (Akunna et al., 1992). The initial biomass concentration in all culture series was 1,468 ± 11 mg VS/L. Incubation of all cultures was carried out in the dark at 35ºC with continuous mixing using a tumbler at 4 rpm.

6.2.2. Kinetics of Nitrate Reduction Assay

Five batch assays were conducted to test the kinetics of nitrate reduction with different electron donors. Each assay was performed using 160-mL serum bottles (112 mL liquid volume) sealed with rubber stoppers and aluminum crimps and pre-flushed with helium gas. An aliquot of 70 mL of the sulfide-acclimated methanogenic culture and 20 mL culture media were anaerobically transferred to each serum bottle. Five different electron donors were used for each assay: D/P, glucose, propionate, acetate, and H_2/CO_2. Each electron donor was added to the serum bottles resulting in an initial chemical oxygen demand (COD) of 1500 mg/L. In order to provide a total of 1500 mg COD/L with a H_2/CO_2 mixture (80% to 20% v/v, respectively), 332 mL of the H_2/CO_2 mixture at 35^{o}C was added to the bottle headspace in increments of approximately 66.4 mL every 30 min.

For each electron donor culture series, one culture was kept nitrate-free and the other ones were amended with nitrate at an initial concentration ranging from 0 to 300 mg N/L using a $NaNO_3$ stock solution. It has been reported that for different nitrate reducing bacterial species, the half velocity coefficient (K_C) value varies between 3.8 to 53 mg N/L (Zumft, 1997). Therefore, the cultures were amended with nitrate concentrations of 0, 5, 10, 25, 50, 75, 150, and 300 mg N/L, i.e., well below and above expected K_C values. Nitrate concentrations lower than 5 mg N/L were not selected due to expected fast nitrate reduction rates, which would have resulted in a limited number of data points. Calculations of stoichiometric quantities of electron donor COD needed relative to the added nitrate concentration were performed as previously described (Tugtas and Pavlostathis, 2007a). Note that for all assays, the electron donor supply was

in excess of that required for the reduction of the added nitrate. Based on the highest initial nitrate concentration added (300 mg NO_3^--N/L) the initial COD provided was 1.75- and 1.10-fold higher than that required for the complete reduction of the added nitrate via denitrification and DNRA, respectively. The initial biomass concentration in all culture series was 1,501± 53 mg VS/L. Incubation of all cultures was carried out in the dark at 35ºC with continuous mixing using a tumbler at 4 rpm.

6.2.3. Kinetic Modeling

The rate of nitrate reduction may be affected by the electron donor type and concentration, as well as toxicity by denitrification intermediates. In addition, concurrent utilization of substrates by fermenters/methanogens and nitrate reducers, may also affect the kinetics of nitrate reduction. The rate of nitrate reduction can be described by a double-substrate Monod equation:

$$-\frac{dC}{dt} = \frac{kXC}{K_C + C}\frac{D}{K_D + D} \qquad (1)$$

where C is the nitrate concentration (mg N/L), k is the nitrate reduction rate per unit biomass (mg/mg VS·d), X is the biomass concentration (mg VS/L), K_C is the half velocity coefficient for nitrate reduction (mg/L), D is the electron donor concentration (mg/L), and K_D is the half velocity coefficient for electron donor utilization (mg/L). As stated above (see Section 6.2.2), the concentration of electron donors was kept in excess compared to the nitrate concentration. All batch assays were consistently conducted with similar initial biomass concentration and the increase in the biomass concentration was negligible (i.e., less than 10%) at the end of the relatively short incubation period in each culture series.

Under these imposed experimental conditions, and because the specific nitrate reducing biomass could not be measured, the rate of nitrate reduction can be described as follows:

$$-\frac{dC}{dt} = \frac{k'C}{K_C + C} \tag{2}$$

where $k' = k\,X$ (mg/L·d) ≈ constant, is the nitrate reduction rate for a constant biomass concentration. Integration of Equation 2 yields

$$C = C_0 - K_C \ln(C/C_0) - k't \tag{3}$$

A nonlinear regression procedure based on the Marquardt-Levenberg algorithm using the SigmaPlot version 8.02 software (Richmond, CA) was used to fit Equation 3 to each nitrate time course data and estimates of k' and K_C values were obtained. However, the accuracy of the K_C values obtained through such regression is questionable, given the fact that obtaining rate data at very low nitrate concentrations was not feasible. K_C values were obtained by an alternative method (see below) which is considered to be more robust. The k' values obtained by the above-described nonlinear regression based on Equation 3, were specific to each initial nitrate concentration, and did not necessarily represent the absolute maximum k' values obtained under nitrate saturation conditions. In order to obtain better K_C estimates, nitrate reduction rate data were plotted versus the initial nitrate concentration and the maximum k' and K_C values were determined using Equation 2 and, in cases where inhibition was observed, the Monod equation with a substrate inhibition term, i.e., the Haldane (Briggs and Haldane, 1925) model:

$$-\frac{dC}{dt} = \frac{k'C}{K_C + C + \frac{C^2}{K_I}} \tag{4}$$

where K_I is the inhibition coefficient (mg/L) and all other parameters as defined above. The inhibition coefficient was included to account for possible inhibitory effects by denitrification intermediates at relatively high nitrate concentrations. Note that a low K_I value indicates the presence of inhibition, whereas a high K_I value indicates absence of any inhibitory effect on the nitrate reduction process.

6.3. Results and Discussion

6.3.1. Preferential Utilization of Electron Donors

The assay testing the preferential utilization of the four electron donors lasted for 8 days. The initial pH value was 7.1 ± 0.02 (mean ± standard deviation) in all four culture series. The final pH values for the nitrate-free/nitrate-amended cultures were 6.9/7.2, 7.2/7.4, 7.3/7.4, and 7.0/7.2 for the D/P-, propionate-, acetate-, and H_2/CO_2-fed cultures, respectively. Methane recovery was monitored and the initial volumetric methane production rates were calculated using linear regression starting at the recovery time (Table 6.1). The initial methane production rate in the nitrate-free cultures was as follows in decreasing order: D/P > H_2/CO_2 > acetate > propionate (Table 6.1). The observed highest methane production in the D/P-fed culture was probably due to the prior acclimation of the culture to this substrate. Acetate and H_2/CO_2 are readily used by methanogens, which contributed to the observed fast initial methane production. Propionate has to go through fermentation and the culture was not exposed to high propionate concentrations before, therefore, the observed slow methane production may be the result of slow utilization of propionate. Addition of nitrate resulted in an immediate suppression of methanogenesis in all four cultures (Figure 6.1B). As a result

Table 6.1. Initial methane production rate and COD utilization in nitrate-free (control) and nitrate-amended mixed methanogenic cultures amended with different electron donors.

Culture series/Electron donor	Methane production			COD Processed (%)			
	Initial rate (mL/L·d)[b]	r^2	Normalized rate (%)[c]	CH_4	VFAs	Nitrate reduction[d]	Total[e]
Nitrate-free cultures							
D/P	21.4 ± 4.7	0.835	100	100	ND	NA[g]	100
Propionate	8.4 ± 0.6	0.985	100	100	ND	NA	100
Acetate	15.9 ± 2.0	0.968	100	100	ND	NA	100
H_2/CO_2	19.7 ± 2.3	0.972	100	100	ND	NA	100
Nitrate-amended cultures[a])							
D/P	10.1 ± 1.7	0.973	47	85	ND	31	116
Propionate	2.1 ± 0.1	0.998	25	34	22	31	87
Acetate	9.7 ± 0.1	0.999	61	79	ND	31	110
H_2/CO_2	ND[f]	ND	ND	ND	ND	31	31

[a] Initial nitrate concentration 50 mg N/L

[b] Results of linear regression (mean ± standard deviation; $n \geq 3$) of single culture data starting at the recovery time.

[c] Normalized to the initial methane production rate of the control culture observed at each culture series.

[d] Fraction of COD utilized for the complete utilization of nitrate to N_2 neglecting microbial growth (calculated).

[e] Normalized to the total COD utilized for methane production in each control culture.

[f] ND, not detected.

[g] NA, not applicable.

of complete utilization of denitrification intermediates, methane production fully recovered in the D/P- and acetate-fed cultures, partially recovered in the propionate-fed culture, and did not recover in the H_2/CO_2-fed culture (Figure 6.1B and C).

The initial rate of methane production in the nitrate-amended cultures fed with different electron donors were as follows in decreasing order: D/P > acetate > propionate (Table 6.1). Stimulation of methanogenesis in the presence of acetate compared to propionate and H_2/CO_2 was reported in nitrate-amended soil microcosms (Roy and Conrad, 1999). Because the initial methane production rate was different for each substrate in the nitrate-free cultures, in order to determine the effect of nitrate addition on methane production, the rates of the nitrate-amended cultures were normalized to the methane production rate in the corresponding nitrate-free culture (Table 6.1). Methanogenesis was affected mostly in the H_2/CO_2-fed culture followed in decreasing order by propionate-, D/P-, and acetate-fed cultures (Table 6.1).

Accumulation of N_2O was observed in the propionate-, acetate-, and H_2/CO_2-fed cultures for 4, 2, and 3 days, respectively (Figure 6.1C). The fastest nitrate reduction rates were observed in the acetate- and H_2/CO_2-fed cultures, followed by D/P- and propionate-fed cultures (Figure 6.2C and D; Table 6.2). However, significant nitrite accumulation was observed in the H_2/CO_2-fed culture. In addition, accumulation of nitric oxide (NO) was observed qualitatively in chromatograms during gas analysis of both the propionate- and H_2/CO_2-fed cultures. The initial rate of N_2 production was as follows in decreasing order: D/P > acetate > propionate > H_2/CO_2 (Table 6.2). Roy and Conrad (1999) also reported stimulation of denitrification mostly by acetate followed by propionate and H_2 in rice field soil microcosms. Slower reduction of the denitrification intermediates

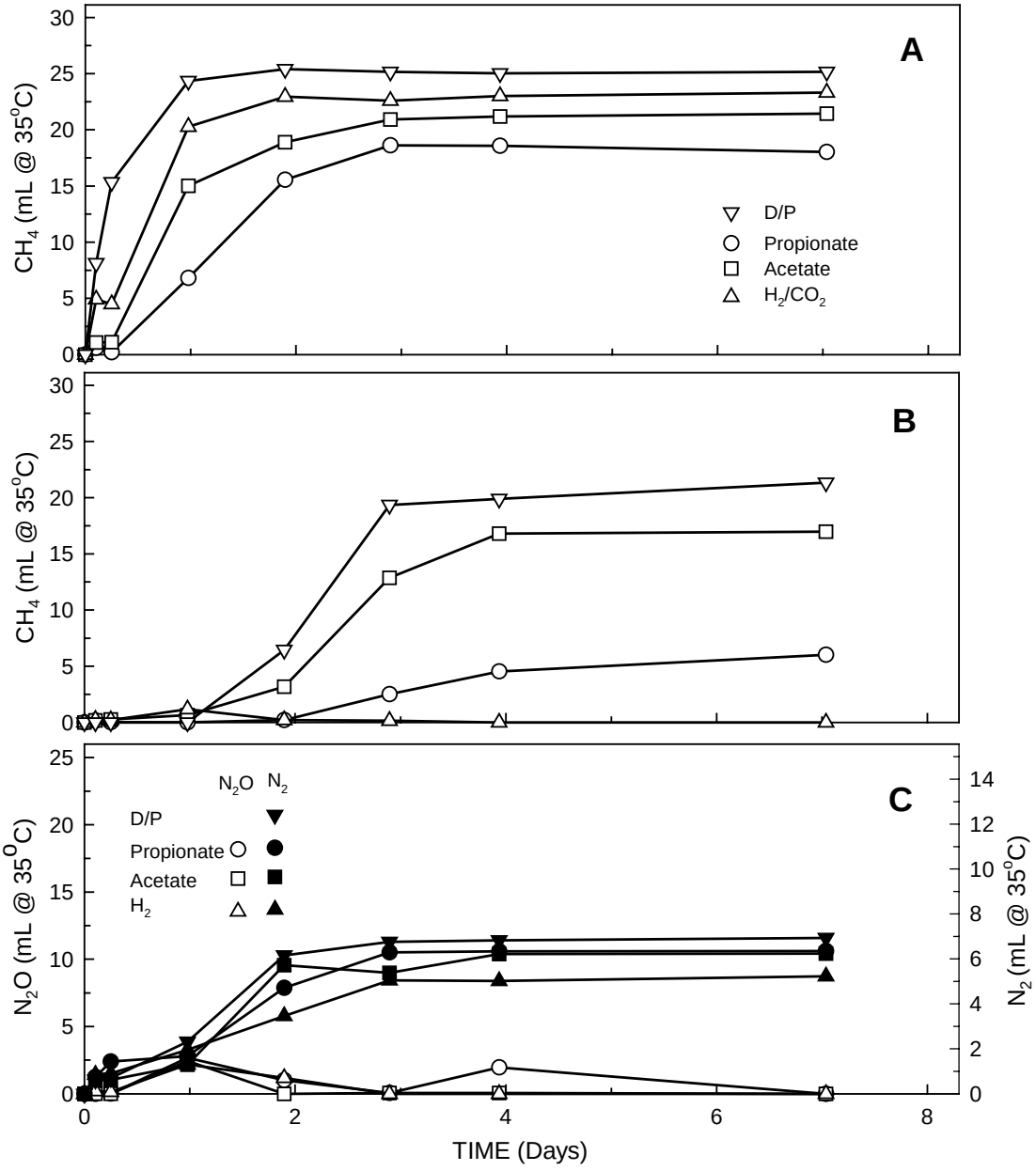

Figure 6.1. Methane production profiles in the nitrate-free (A), nitrate-amended (B) cultures, and production and consumption of N_2O and production of N_2 in the nitrate-amended cultures (C).

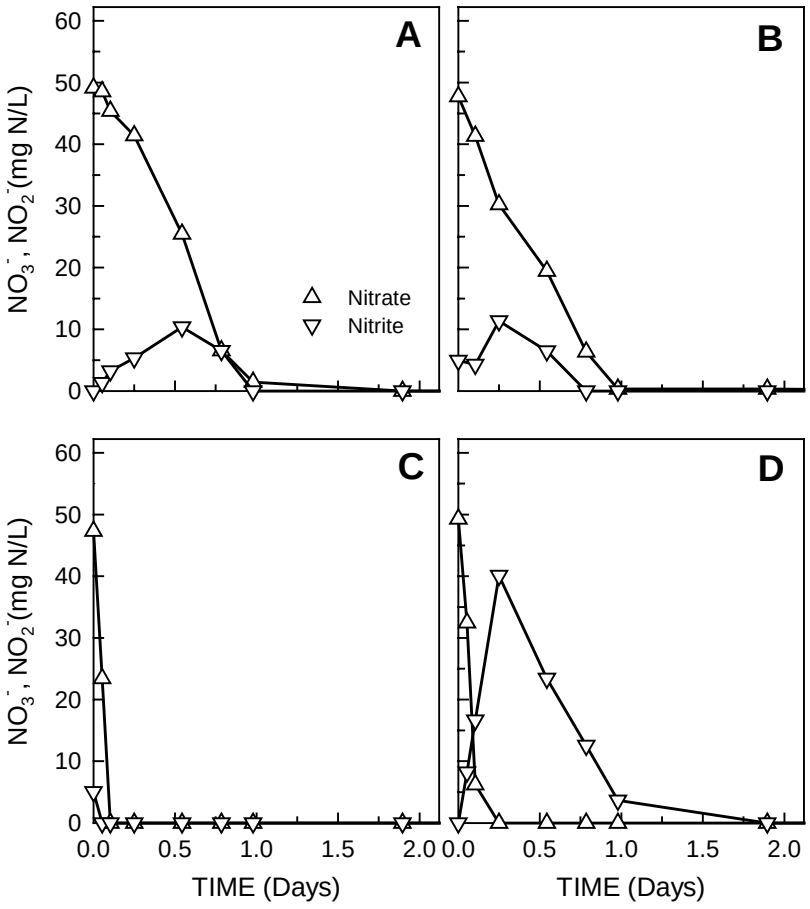

Figure 6.2. Nitrate and nitrite profiles in D/P- (A), propionate- (B), acetate- (C), and H_2/CO_2-fed (D) cultures.

Table 6.2. Nitrate reduction and N_2 production rates in methanogenic cultures fed with different types of electron donors[a].

	Nitrate Reduction		N_2 Production	
Electron donor	*k'* (mg NO_3^--N/L·d)	r^2	Initial rate (mL/L·d)	r^2
D/P	55.6 ± 3.5	0.987	3.1 ± 0.3	0.962
Propionate	51.3 ± 3.4	0.992	2.0± 0.2	0.892
Acetate	456.2 ± 0.1	0.999	3.2± 1.1	0.986
H_2/CO_2	282.0 ± 0.2	0.882	1.6 ± 0.1	0.973

[a] Mean ± standard error ($n \geq 3$)

associated with the type of the electron donor, could be the reason for slower N_2 production in the cultures in which the fastest nitrate reduction was observed (Table 6.2). Acetate and propionate production was observed in the D/P-fed culture (Figure 6.3A). Slightly higher acetic acid production was observed in the nitrate-amended, D/P-fed culture as opposed to the control culture. The COD required for the complete nitrate reduction was calculated based on 2.857 mg COD/mg NO_3^--N (Table 6.1). The total processed COD in the D/P-fed, nitrate-amended culture was similar to that of the nitrate-free control culture, suggesting that all of the initial COD was utilized by nitrate reducers, fermenters, and methanogens (Table 6.1). Although the propionate utilization profiles were the same in both the nitrate-free and nitrate-amended, propionate-fed cultures at the beginning of the incubation, the propionate utilization rate became slower concomitant to complete cessation of nitrate reduction. Approximately 22% of the initially added COD remained as propionate at the end of the incubation (Figure 6.3B and Table 6.1). As mentioned above, the methane production recovered only partially in the nitrate-amended, propionate-fed culture accounting for only 34% of the initial COD and 13% of the initial COD remained unutilized (Table 6.1). In addition, acetate production was observed as a result of cessation of nitrate reduction suggesting that propionate was directly utilized by nitrate reducers. The acetate utilization profiles were similar in both the nitrate-free and nitrate-amended, acetate-fed cultures (Figure 6.3C). At the time when complete nitrate reduction occurred, acetate utilization slowed down, which is attributed to the cessation of acetate utilization by denitrifiers (Figures 6.2C and 6.3C). The total processed COD was close to that of the acetate-fed, nitrate-free culture (Table 6.1). In the H_2/CO_2-fed culture, 31% of the initially added COD was utilized for nitrate reduction and

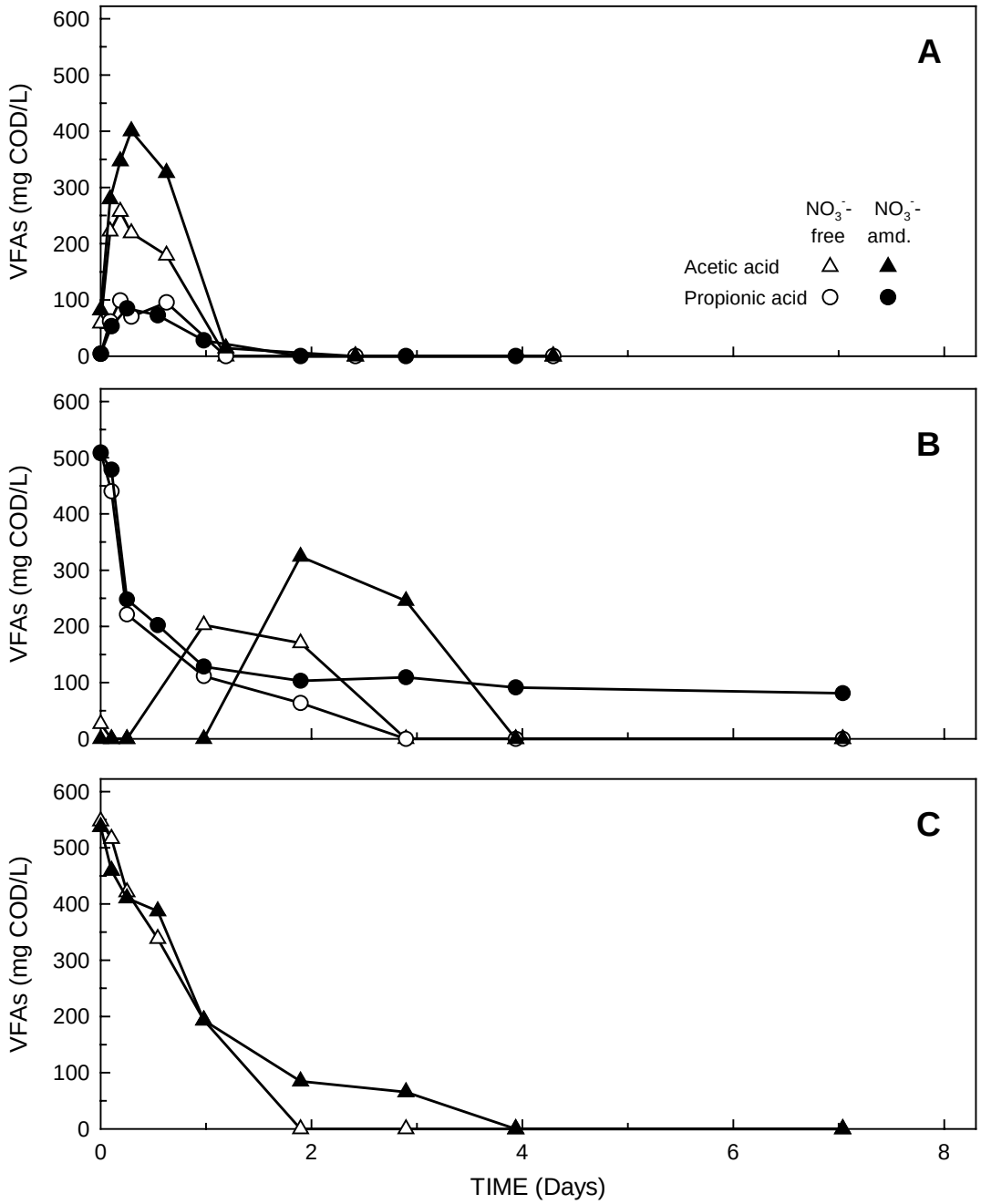

Figure 6.3. VFA production and consumption profiles in the cultures fed with D/P (A), propionate (B), and acetate (C). VFAs were not measured in the H_2/CO_2-fed cultures.

the remainder was not consumed due to complete inhibition of methanogenesis as a result of accumulation of denitrification intermediates (Table 6.1).

In summary, the utilization of different electron donors by nitrate reducers within a methanogenic system results in accumulation of different levels of denitrification intermediates, which in turn have a different impact on methanogenesis (i.e., varying from complete inhibition to full recovery).

6.3.2. Nitrate Reduction Kinetics with Different Electron Donors

The assays testing the effect of different electron donors on nitrate reduction lasted for 7 days. The initial pH value was 7.3 ± 0.1 (mean ± standard deviation) in all five culture series. The initial ammonia concentration was 186 ± 12, 182 ± 23, 178 ± 32, 181 ± 17, and 169 ± 13 in the D/P-, glucose-, propionate-, acetate-, and H_2/CO_2-fed cultures, respectively. The nitrate and nitrite data for all five culture series are shown in Figures 6.4 and 6.5. The highest nitrite accumulation was observed in the propionate and H_2/CO_2-fed cultures for all the applied nitrate concentrations (Figure 6.5). Figure 6.6 shows the nitrate and nitrite data, along with the model fit (Equation 3) to the nitrate data, of the 300 mg N/L nitrate-amended cultures as a representative of all other nitrate-amended cultures. Nitrate reduction at an initial nitrate concentration of 300 mg N/L occurred approximately in 2 days in the D/P- and glucose-fed cultures, in 5 days in the propionate-fed culture, and in less than 12 h in the acetate- and H_2/CO_2-fed cultures (Figure 6.6).

Nitrate reduction took place mainly via the denitrification pathway in the propionate-, acetate-, and H_2/CO_2-fed cultures, whereas in the D/P- and glucose-fed

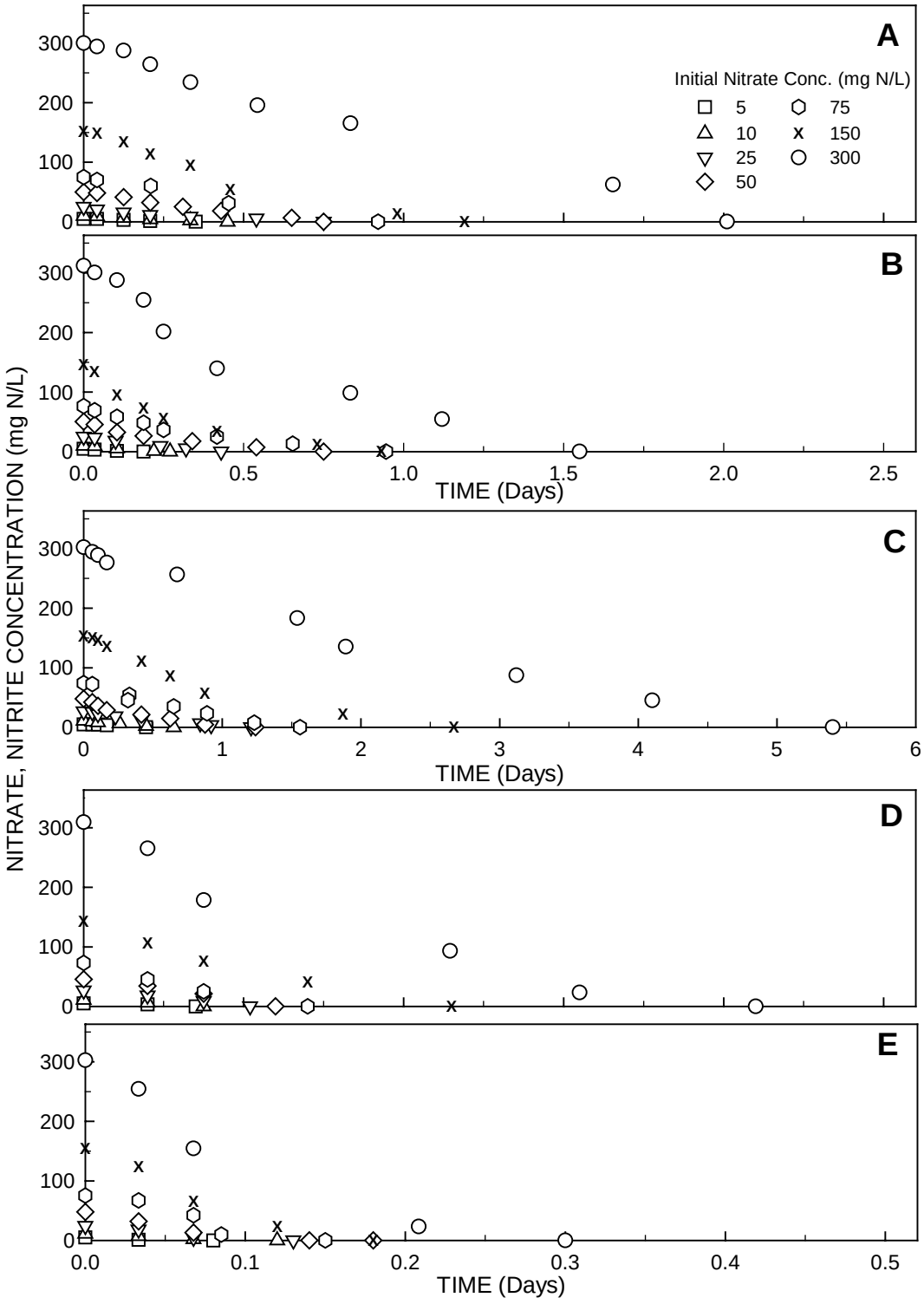

Figure 6.4. Experimental data of nitrate profiles in (A) D/P-, (B) glucose-, (C) propionate-, (D) acetate-, and (E) H_2/CO_2-fed cultures amended with 5 to 300 mg N/L nitrate.

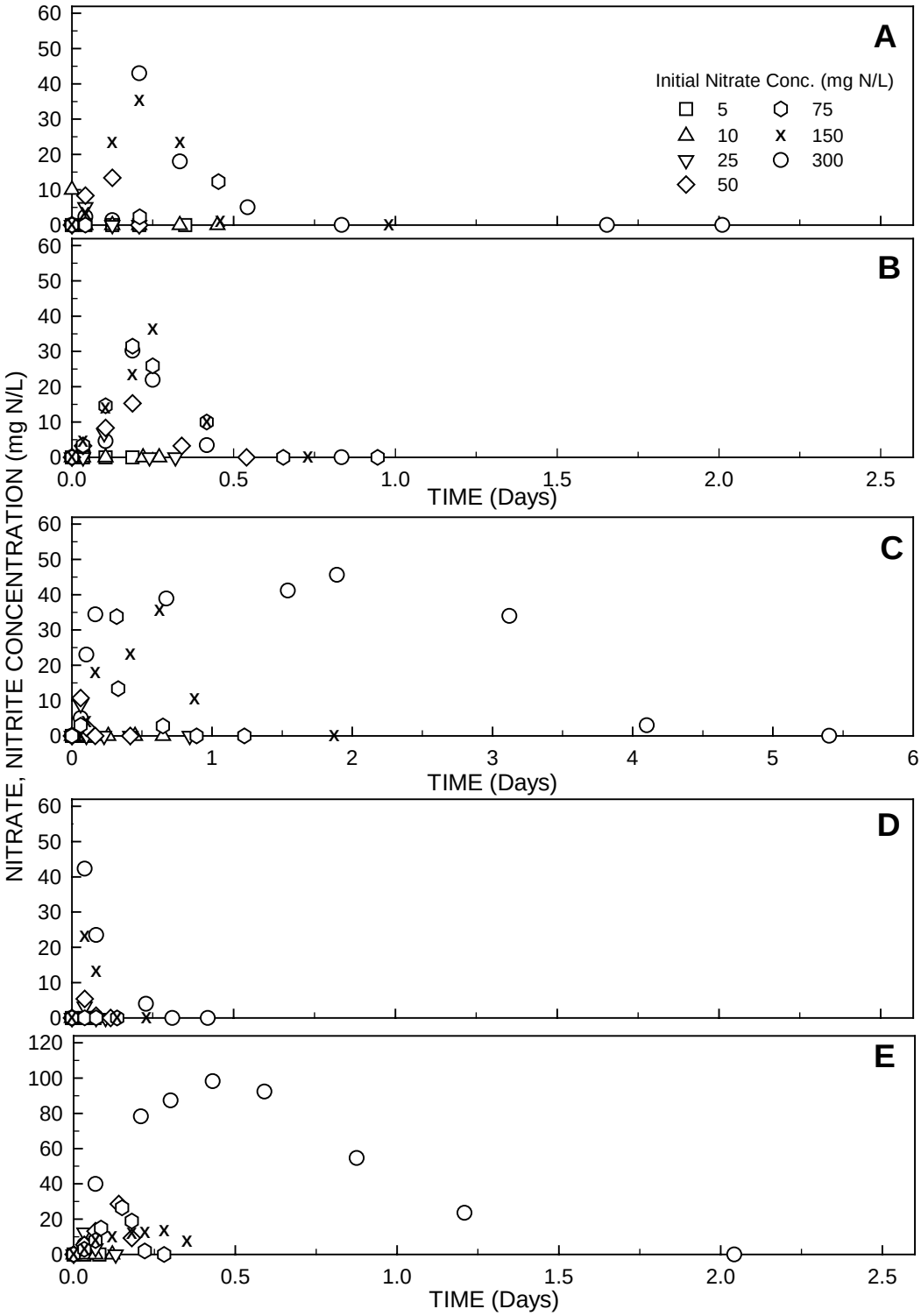

Figure 6.5. Experimental data of nitrite profiles in (A) D/P-, (B) glucose-, (C) propionate-, (D) acetate-, and (E) H_2/CO_2-fed cultures amended with 5 to 300 mg N/L nitrate. Lines are model predictions.

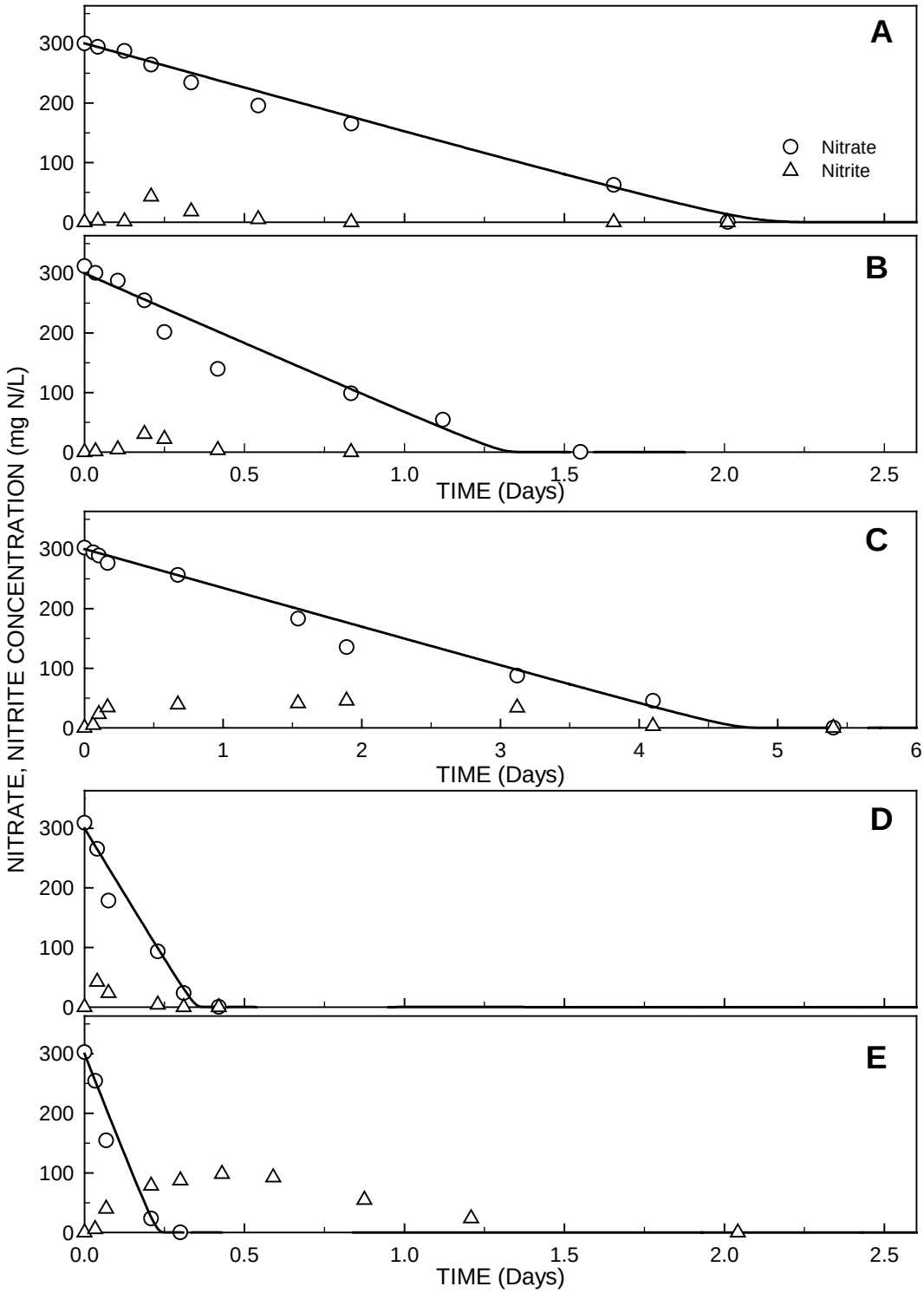

Figure 6.6. Experimental data of nitrate and nitrite profiles in (A) D/P-, (B) glucose-, (C) propionate-, (D) acetate-, and (E) H_2/CO_2-fed cultures amended with 300 mg N/L nitrate. Lines are model predictions.

cultures, nitrate was reduced via both the denitrification and DNRA pathways (Tables 6.3 and 6.4). Approximately similar levels of methane were measured at the end of the incubation in the D/P-, glucose-, and acetate-fed cultures for all the nitrate-amendments (Table 6.3). Figure 6.7 shows the methane and N_2 data for only the 300 mg N/L nitrate-amended cultures as a representative of all other nitrate-amended cultures. In addition, the total processed COD in the D/P-, glucose-, and acetate-fed cultures was very close to that of the nitrate-free control culture, indicating that all initially supplied COD was used for denitrification, DNRA and methane production (Table 6.3). Methane production recovered only partially in the propionate-fed cultures and VFA accumulation was observed at the end of the incubation in cultures amended with 10 mg N/L nitrate or higher (Table 6.3). Methane production did not recover and a very slow N_2 production was observed in the 300 mg N/L nitrate-amended, propionate-fed culture (Figure 6.7). NO accumulation was observed in the propionate-fed cultures during the incubation, which might be the reason for the inhibition of fermenters and methanogens resulting in the accumulation of VFAs at the end of the incubation. The processed COD was significantly less in the 10 to 150 mg N/L nitrate-amended, propionate-fed cultures than in the control culture (Table 6.3). The unaccounted amount of COD is presumed to be either unutilized COD or products not analyzed for with the methods used in this study (Table 6.3).

Similarly to the propionate-fed cultures, methane production either partially recovered or did not recover in the H_2/CO_2-fed, nitrate-amended cultures (Figure 6.7A and Table 6.3). However, during the incubation, accumulation of nitrite as well as NO was observed in the H_2/CO_2-fed cultures, which inhibited the methanogens (Figures 6.6

Table 6.3. COD utilization and products at the end of incubation in mixed methanogenic cultures fed with different substrates and amended with different initial nitrate conc's.

Substrate	Initial Nitrate Conc. (mg N/L)	CH_4 (mL)	N_2 (mL)	VFAs (mg COD/L)	NH_4^+ (mg N/L)[b]	COD Processed (%)				
						CH_4	VFAs	Nitrate reduction via DNRN[c]	Nitrate reduction via DNRA[d]	Total[e]
D/P	0	53.3	ND	ND	ND	100	0	0		100
	5	52.7	0.4	ND	1.1	98.9	0	0.9	0.3	100.1
	10	50.1	0.5	ND	5.5	94.0	0	1.1	1.7	96.8
	25	49.6	0.4	ND	21.1	93.1	0	0.9	6.4	100.4
	50	46.2	1.3	ND	36.7	86.7	0	3.2	11.2	101.1
	75	40.5	2.3	ND	52.5	76.0	0	5.4	16.0	97.4
	150	33.8	12.4	ND	27.3	63.4	0	29.5	8.3	101.2
	300	14.3	29.7	ND	6.2	26.9	0	70.6	1.9	99.5
Glucose	0	51.9	ND	ND	ND	100	0	0	0	100
	5	48.4	0.2	ND	2.9	93.3	0	0.5	0.9	94.6
	10	46.5	0.4	ND	6.4	89.6	0	0.9	1.9	92.4
	25	44.1	0.7	ND	18.2	85.0	0	1.6	5.5	92.2
	50	38.6	2.4	ND	26.3	74.4	0	5.7	8.0	88.1
	75	37.5	4.1	ND	34.4	72.3	0	9.8	10.5	92.5
	150	25.4	13.8	ND	13.2	48.9	0	32.9	4.0	85.9
	300	12.3	30.8	ND	ND	23.7	0	73.2	0	96.9
Propionate	0	49.8	ND	ND	ND	100	0	0	0	100
	5	42.3	0.6	ND	ND	91.0	0	1.5	0	92.5
	10	38.2	1.1	10.9	ND	76.7	7.3	2.6	0	86.5
	25	24.3	2.1	24.6	ND	48.4	16.4	5.1	0	70.3
	50	17.5	5.2	43.5	ND	35.1	29.0	12.4	0	76.5
	75	8.3	6.9	58.2	ND	16.7	38.8	16.4	0	71.8
	150	ND[a]	15.2	80.9	ND	0.0	53.9	36.1	0	90.1
	300	ND	31.4	45.0	ND	0.0	30.3	74.7	0	104.7
Acetate	0	52.7	ND	ND	ND	100	0	0	0	100
	5	51.9	0.5	ND	ND	98.5	0	1.3	0	99.8
	10	51.1	1.1	ND	ND	97.0	0	2.7	0	99.6
	25	48.3	2.7	ND	ND	91.7	0	6.5	0	98.2
	50	42.0	5.8	ND	ND	79.7	0	13.8	0	93.5
	75	38.2	7.9	ND	ND	72.5	0	18.8	0	91.3
	150	31.3	16.1	ND	ND	59.4	0	38.4	0	97.8
	300	13.1	29.9	ND	ND	24.9	0	71.2	0	96.0
H_2/CO_2	0	51.2	ND	ND	ND	100	0	0	0	100
	5	48.4	0.5	ND	ND	94.5	0	1.2	0	95.7
	10	27.4	1.1	ND	ND	53.5	0	2.6	0	56.1
	25	8.3	2.8	ND	ND	16.2	0	6.7	0	22.9
	50	ND	5.2	ND	ND	0	0	12.4	0	12.4
	75	ND	7.4	ND	ND	0	0	17.7	0	17.7
	150	ND	14.7	ND	ND	0	0	35.1	0	35.1
	300	ND	30.2	ND	ND	0	0	71.9	0	71.9

[a] ND, not detected.

[b] Net NH_4^+ production at the end of the incubation (i.e., corrected for the inoculum initial NH_4^+ contribution; see text).

[c] Fraction of COD utilized for the complete reduction of nitrate to N_2 (dissimilatory nitrate reduction to nitrogen gas; DNRN), neglecting microbial growth (see text).

[d] Fraction of COD utilized for the complete reduction of nitrate to NH_4^+, neglecting microbial growth (see text).

[e] Normalized to the total COD utilized for methane production in the control (i.e., nitrate-free) culture.

and 6.7A). The processed COD was significantly lower in the H_2/CO_2-fed, nitrate-amended cultures than in the control culture. The unaccounted amount of COD is presumed to be unutilized H_2 as a result of inhibition of methanogens (Table 6.3).

The processed COD for denitrification and DNRA was calculated based on 2.854 mg COD/mg NO_3^--N reduced to N_2 and 4.57 mg COD/mg NO_3^--N reduced to NH_4^+ and results are shown in Table 6.3. Then, the fraction of nitrate reduced via the denitrification and/or the DNRA pathway was calculated based on the volume of N_2 and the net NH_4^+ produced in the nitrate-amended cultures and results are shown in Table 6.4. For low initial nitrate concentrations (i.e., lower than 10 mg N/L), the total fraction of nitrate converted to N_2 and/or NH_4^+ was above 100% (Table 6.4), which is attributed to the analytical difficulty of measuring very low levels of N_2. In general, DNRA was the dominant pathway of nitrate reduction in the D/P- and glucose-fed cultures at COD/N values higher than 20 and 60, respectively (Table 6.4). Akunna et al. (1992) also reported that DNRA became the main pathway of nitrate reduction at COD/N values greater than 53 in digested sludge cultures. As mentioned above, DNRA was not observed in cultures fed with propionate-, acetate-, or H_2/CO_2, and the main pathway of nitrate reduction in these cultures was denitrification, suggesting that the predominance of DNRA over denitrification was mainly the result of the electron donor type rather than the COD/N value (Table 6.4).

The nitrate profiles obtained by using Equation 3 and the estimated values of k' and K_C agree well with the experimental data (Figure 6.6). The measured nitrate reduction rates in the presence of different electron donors and at a range of initial nitrate concentrations are reported in Table 6.5. The lowest nitrate reduction rates were observed

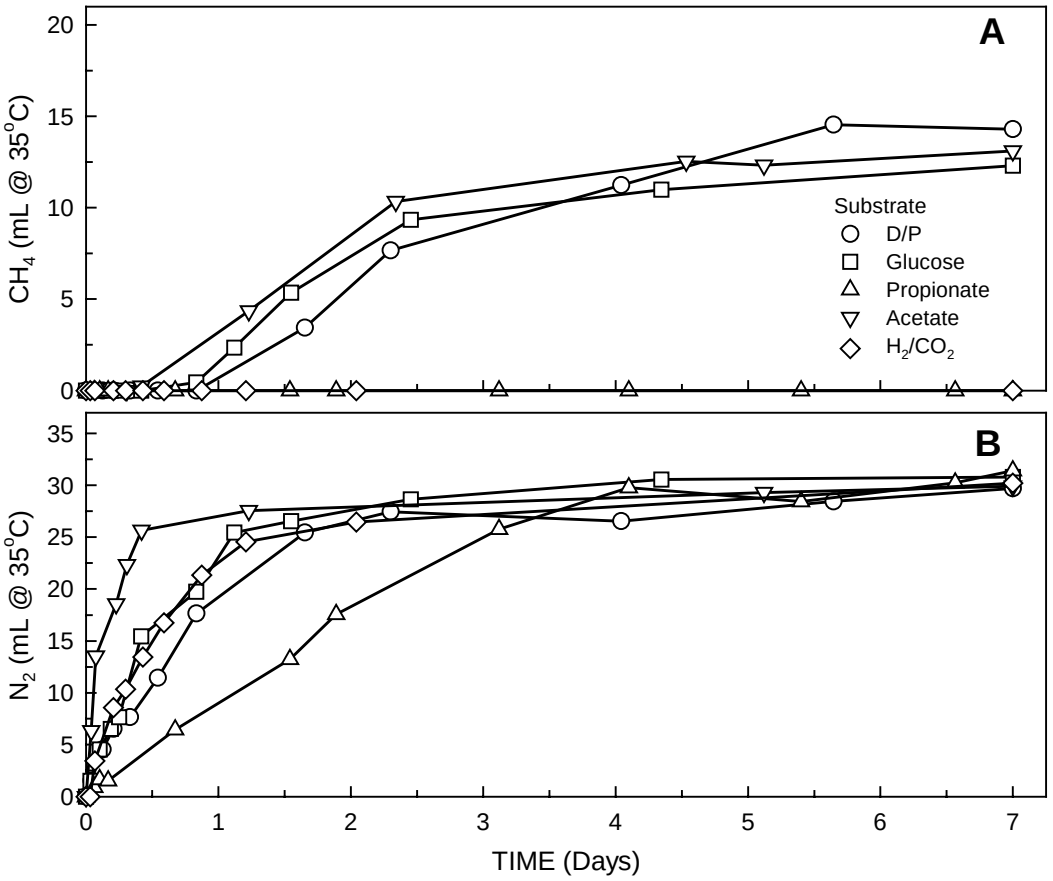

Figure 6.7. (A) Methane and (B) N_2 gas production profiles for all cultures fed with different electron donors and amended with an initial nitrate concentration of 300 mg N/L.

Table 6.4. Fraction of nitrate reduced to NH_4^+ (DNRA) and/or N_2 (DNRN) as a function of substrate type and initial nitrate concentration in a mixed methanogenic culture[a].

Initial NO_3^--N Conc. (mg/L)	COD/N	D/P		Glucose		Propionate		Acetate		H_2/CO_2	
		DNRA (%)	DNRN (%)	DNRA (%)	DNRN (%)	DNRA (%)	DNRN (%)	DNRA (%)	DNRN (%)	DNRA (%)	DNRN (%)
5	300	23	77	57	48	ND	123	ND	106	ND	101
10	150	55	45	64	36	ND	107	ND	111	ND	108
25	60	84	16	73	27	ND	85	ND	108	ND	112
50	30	73	27	53	48	ND	103	ND	115	ND	103
75	20	70	30	46	54	ND	91	ND	104	ND	98
150	10	18	82	9	91	ND	100	ND	107	ND	97
300	5	2	98	ND[b]	102	ND	104	ND	99	ND	100

[a] Based on nitrogen balance calculations (see text).
[b] ND, not detected.

for the propionate-fed cultures, suggesting that propionate was the least preferentially utilized substrate by nitrate reducers among the other substrates used in this study (Table 6.5). The nitrate reduction rates in the cultures fed with different substrates were as follows in descending order: H_2/CO_2 > acetate > glucose > D/P > propionate. The slow nitrate reduction in the D/P-fed culture was presumably the result of D/P hydrolysis and/or fermentation, which may have been the rate-controlling step(s). The fastest and slowest N_2 gas production occurred in the acetate-fed and propionate-fed, 300 mg N/L nitrate-amended cultures, respectively (Figure 6.7B).

The measured nitrate reduction rates were plotted against the initial nitrate concentration as shown in Figure 6.8. Inhibition was observed in the D/P- and propionate-fed cultures at initial nitrate concentrations higher than 150 mg N/L (Figure 6.8A and C). Therefore, the values of k', K_C and K_I for each electron donor were estimated using non-linear regression based on Equations 2 and 4 (i.e., Monod and Haldane equation, respectively) and results are shown in Table 6.6. Although the Haldane equation described the nitrate reduction pattern in the D/P- and propionate-fed cultures very well, the error estimates were on the order of the mean estimates because of the limited number of data points at initial nitrate concentrations equal and higher than 150 mg N/L. Because inhibition was not observed in the glucose-, acetate-, and H_2/CO_2-fed cultures, both the Monod and Haldane equations fit the data very well (Figure 6.8B, D, and E). For the glucose-, acetate-, and H_2/CO_2-fed cultures, the estimated K_I values were extremely high (> 10^7 mg/L), which suggests that the nitrate levels used in this study were not inhibitory in the presence of these three substrates

Table 6.5. Nitrate reduction rates (*k'*) in mixed methanogenic cultures fed with different electron donors and amended with a range of initial nitrate concentrations.[a]

Initial Nitrate Conc. (mg N/L)	*k'* (mg NO_3^--N/L·d) [*k*, mg N/mg VS·d]				
	D/P	Glucose	Propionate	Acetate	H_2/CO_2
5	19 ± 3 [0.012 ± 0.002]	36 ± 4 [0.024 ± 0.003]	12 ± 4 [0.008 ± 0.003]	73 ± 4 [0.049 ± 0.003]	134 ± 5 [0.09 ± 0.003]
10	25 ± 2 [0.017 ± 0.001]	42 ± 4 [0.028 ± 0.003]	19 ± 1 [0.013 ± 0.0006]	146 ± 10 [0.097 ± 0.006]	138 ± 13 [0.092 ± 0.009]
25	35 ± 7 0.023 ± 0.005]	64 ± 2 [0.042 ± 0.001]	25 ± 2 [0.017 ± 0.001]	255 ± 38 [0.17 ± 0.025]	311 ± 77 [0.21± 0.05]
50	70 ± 4 [0.046 ± 0.003]	79 ± 12 [0.052 ± 0.008]	47 ± 6 [0.031 ± 0.004]	382 ± 83 [0.25 ± 0.06]	533 ± 25 [0.355 ± 0.017]
75	96 ± 11 [0.064 ± 0.007]	99 ± 12 [0.066 ± 0.008]	55 ± 5 [0.036 ± 0.003]	648 ± 36 [0.432 ± 0.024]	734 ± 234 [0.49 ± 0.16]
150	155 ± 18 [0.103 ± 0.012]	186 ± 38 [0.12 ± 0.03]	78 ± 10 [0.052 ± 0.006]	740 ± 82 [0.49 ± 0.05]	1169 ± 135 [0.78 ± 0.09]
300	152 ± 9 [0.101 ± 0.006]	237 ± 31 [0.158 ± 0.02]	66 ± 4 [0.044 ± 0.003]	898 ± 124 [0.59 ± 0.08]	1389 ± 250 [0.93 ± 0.17]

[a] Mean ± standard error ($n \geq 5$; $r^2 \geq 0.892$)

Table 6.6. Maximum nitrate reduction rates (k' and k), half velocity coefficient (K_C) and inhibition coefficient (K_I) values estimated using the Monod and Haldane equations as a function of initial nitrate concentration.

Substrate	Michaelis–Menten			Haldane			
	k' (mg N/L·d) [k, mg N/mg	K_C (mg N/L)	r^2	k' (mg N/L·d) [k, mg N/mg	K_C (mg N/L)	K_I (mg N/L)	r^2
D/P	212 ± 25 [0.08 ± 0.008]	89 ± 25	0.965	377 ± 238 [0.25 ± 0.16]	193 ± 153	137 ± 128	0.989
Glucose	358 ± 57 [0.24 ± 0.04]	153 ± 49	0.962	358 ± 54 [0.24 ± 0.04]	154 ± 54	NA[a]	0.950
Propionate	84 ± 8 [0.06 ± 0.005]	40 ± 12	0.952	219 ± 142 [0.15 ± 0.09]	171 ± 143	180 ± 185	0.970
Acetate	1139 ± 88 [0.76 ± 0.06]	78 ± 15	0.983	1139 ± 97 [0.76 ± 0.06]	78 ± 17	NA	0.964
H_2/CO_2	2036 ± 129 [1.36 ± 0.09]	129 ± 18	0.993	2036 ± 141 [1.36 ± 0.09]	129 ± 191	NA	0.993

[a] NA, not applicable (i.e., very high K_I values; see text)

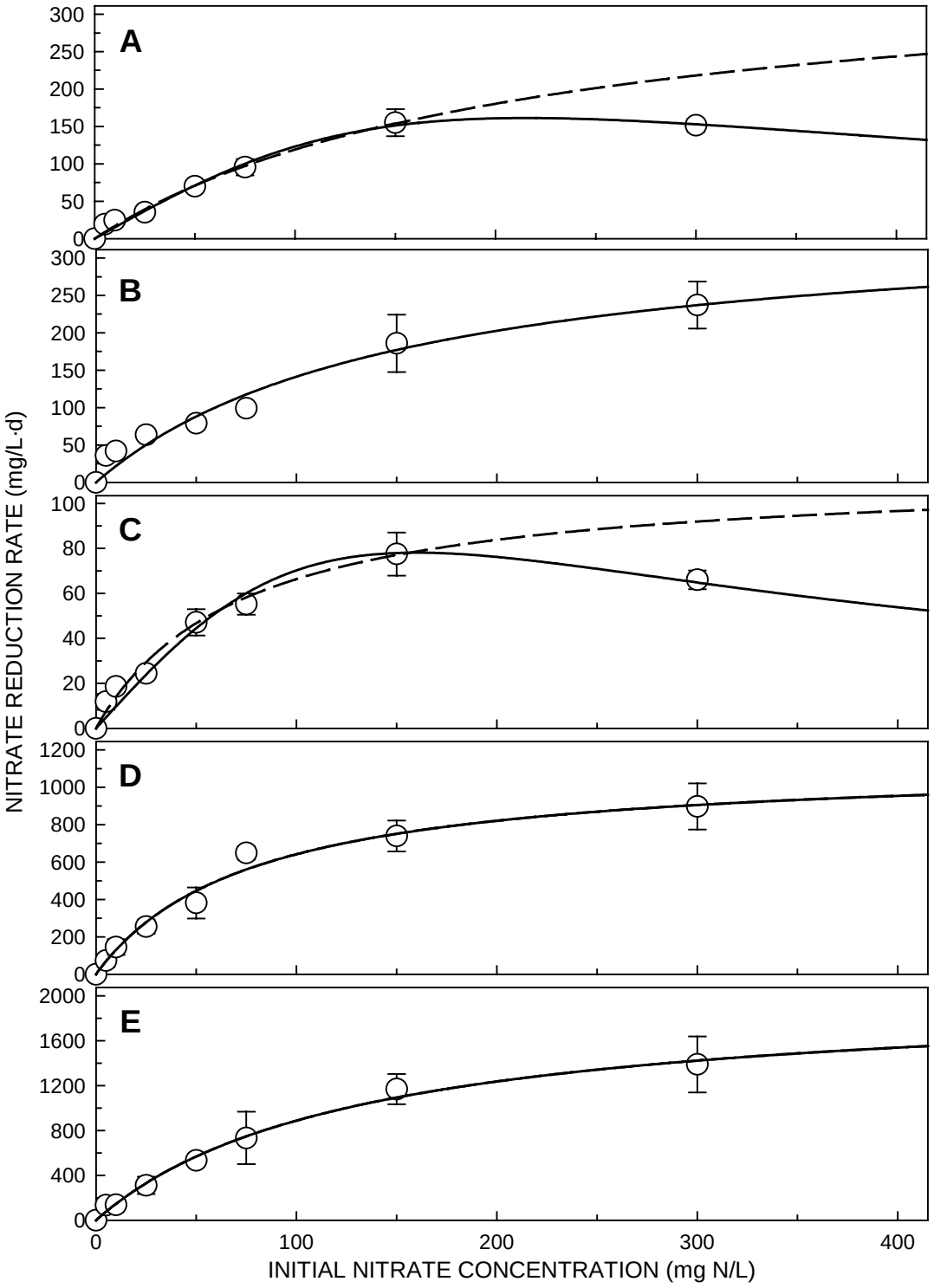

Figure 6.8. Nitrate reduction rates as a function of initial nitrate concentration for (A) D/P-, (B) glucose-, (C) propionate-, (D) acetate-, and (E) H_2/CO_2-fed mixed, methanogenic cultures. Error bars represent mean values ± standard error (n = 3). Solid lines represent fits based on the Monod equation (panel B, D, and E), and Haldane equation (panel A, and C). Dashed lines represent fits based on the Monod equation (panel A and C).

As a result, both the Monod and Haldane equations resulted in similar k' and K_C values for these three culture series (Table 6.6).

The biomass-normalized nitrate reduction rate values (i.e., k) ranged between 0.06 and 1.36 mg N/mg VS·d for the different electron donors used (Table 6.6). Considering that the total biomass concentration was used in these calculations, as opposed to the concentration of nitrate reducers, which were a fraction of the total biomass, the biomass-normalized nitrate reduction rate estimates reported here are conservative values. In a study conducted with anaerobic sludge, Akunna et al. (1993) reported nitrate reduction rates of 19.4 and 200.1 mg/L·d at an initial nitrate concentration of 200 mg N/L in the presence of glucose and acetate, respectively. Based on the biomass concentration in the Akunna et al. (1993) study (100 mg VSS/L), their biomass-normalized nitrate reduction rates were 0.19 and 2.0 mg N/mg VSS·d. Nitrate reduction rates of 0.12 ± 0.03 and 0.49 ± 0.05 mg N/mg VS·d were observed in the glucose- and acetate-fed, 150 mg N/L nitrate-amended cultures, respectively (Table 6.5). Although the initial nitrate concentrations were slightly different, similar rates in the glucose and lower rate in the acetate-fed cultures were observed in the present study as compared to the values reported by Akunna et al. (1993). A nitrate reduction rate of 0.76 mg N/mg VSS·d was reported for glucose-fed, pure cultures of *Bacillus subtilis* under anoxic conditions (Marazioti et al., 2003). The reported nitrate reduction rates in the presence of H_2 for pure cultures of *Acidovorax sp.* and *Acinetobacter sp.* were 1.82 and 0.215 mg N/mg VSS·d, respectively (Vasiliadou et al., 2006), which are comparable to the values obtained in this study. Nitrate reduction rates of 0.03 to 0.05 mg N/mg VS·d at were reported for initial nitrate concentrations of 50.8 to 204.3 mg N/L in acid phase digester sludge when mixed VFAs

were used as substrate (Elefsiniotis et al., 2004). The nitrate reduction rates in the present study were comparable to those reported by Elefsiniotis et al., 2004. The reported K_C values for nitrate reduction vary from microorganism to microorganism (Betlach and Tiedje, 1981) and are generally in the range of 3 x 10^{-5} to 53 mg N/L (Zumft, 1997). The K_C values obtained in this study were higher than those reported in the literature. Higher half velocity (K_C) values typically reflect mass transfer limitations, i.e., are apparent values as opposed to intrinsic values obtained in the absence of any mass transfer limitation (Pavlostathis, 2006). Therefore, the relatively higher K_C values obtained in the present study may be the result of mass transfer limitations.

6.4. Summary

The preferential utilization of different electron donors and their effect on the pathway and kinetics of nitrate reduction in a sulfide-acclimated mixed, mesophilic (35^{o}C) methanogenic culture were investigated. In order to determine the preferential utilization of different carbon sources, cultures were fed with D/P-, propionate-, acetate-, and H_2/CO_2- at an initial COD of 500 mg N/L and amended with nitrate at an initial concentration of 50 mg N/L. Immediate cessation of methane production was observed in all the nitrate-amended cultures. Methane production completely recovered in the D/P- and acetate-fed cultures, and partially recovered or did not recover in the propionate- and H_2/CO_2-fed, nitrate-amended cultures, respectively. Accumulation of denitrification intermediates was observed in both the propionate- and H_2/CO_2-fed cultures, which resulted in inhibition of fermentation and/or methanogenesis. The fastest and the slowest nitrate reduction rates were observed in the acetate-fed and propionate-fed cultures.

In order to delineate the kinetics of nitrate reduction in the presence of different electron donors, D/P, glucose, propionate, acetate, and H_2/CO_2 were used as substrates at an initial concentration of 1500 mg COD/L and the initial nitrate concentration ranged between 0 and 300 mg N/L. The fastest nitrate reduction was observed in the H_2/CO_2 and acetate-fed cultures. In the case of propionate, nitrate reduction was the slowest followed by partial recovery of methanogenesis and accumulation of VFAs due to inhibition as a result of accumulation of denitrification intermediates. Similarly, accumulation of nitrite and nitric oxide and partial or complete inhibition of methanogenesis was observed in the H_2/CO_2-fed cultures. Methanogenesis completely recovered in the dextrin/peptone-, glucose-, and acetate-fed cultures at all nitrate levels. Denitrification was the dominant pathway of nitrate reduction in the propionate-, acetate-, and H_2/CO_2-fed cultures regardless of the COD/N value. However, both denitrification and DNRA were observed in the dextrin/peptone- and glucose-fed cultures and the predominance of either of the two pathways was a function of the COD/N value. Therefore, the type of electron donor used affected both the nitrate reduction pathway and kinetics, as well as the recovery of fermentation and/or methanogenesis in the mixed methanogenic culture.

CHAPTER 7

MODELING OF SIMULTANEOUS NITRATE REDUCTION AND METHANOGENESIS PROCESSES

7.1. Introduction

Although several models have been developed in order to describe the simultaneous nitrate reduction and methanogenesis (Chaudhry and Beg, 1997; Garibay-Orijel et al., 2006), a comprehensive model considering substrate competition and inhibition does not exist.

The objective of the research described here was to incorporate the effect of nitrate reduction on fermentation/methanogenesis into the IWA Anaerobic Digestion Model No. 1 (ADM1) in order to simulate such process interactions within a complex anaerobic system receiving both an organic wastewater and nitrate. The results of the extended ADM1 were compared to experimental data obtained from a series of batch experiments conducted with a mixed fermentative/methanogenic culture fed with a mixture of dextrin and peptone and amended with various initial nitrate concentrations. Model simulations were also used in order to determine the effect of inhibition by N-oxides and the fraction of the denitrifying biomass on nitrate reduction and fermentation/methanogenesis. In addition, model simulations were used to investigate the effect of nitrate on methanogenesis in continuous-flow systems at different influent nitrate concentrations, when a step increase in influent nitrate concentration occurs, and when a step decrease in SRT of the system occurs.

7.2. Model Development

7.2.1. Modification of Disintegration and Hydrolysis

The feed composition in the extended ADM1 was adjusted to reflect the culture conditions described in Chapter 3. Upon disintegration, the decayed biomass was assumed to result in 80% biodegradable (10.4% carbohydrates, 66.4% proteins, and 3.2% lipids) and 20% inert material (10% soluble and 10% particulate) according to previously reported results on the fate of biological solids in anaerobic digestion systems (Pavlostathis, 1985). In the ADM1 report, the same hydrolysis rate constant value was suggested for both the influent feed and decayed biomass (Table 7.1). However, it has been demonstrated that hydrolysis of dead biomass was the slowest step in the overall anaerobic digestion process (Pavlostathis, 1985). Therefore, in the modified model, hydrolysis of the feed and the decayed biomass were considered as two separate processes with different rate constants, as follows: a) decayed biomass: disintegration rate, 2 d^{-1}; hydrolysis rate for carbohydrates, proteins, and lipids, 0.15, 0.5, and 0.15 d^{-1}, respectively; b) feed: there is no disintegration step for dextrin and peptone; the hydrolysis rate for both carbohydrates and proteins was set equal to 2 d^{-1}. In addition, acetate and propionate utilization rates, equal to 6 and 5.6 mg substrate-COD/mg biomass-COD · d, calculated based on bioenergetic principles, were used in the model. The K_s value for propionate utilization was increased from 100 to 500 mg substrate-COD/L as a result of model calibration using experimental data.

7.2.2. Extension of the ADM1 with Denitrification Process

As discussed in Chapter 5 nitrate reduction proceeds either via denitrification and/or dissimilatory nitrate reduction to ammonia (DNRA). The extended model describes denitrification, i.e., the sequential reduction of nitrate to nitrite, nitrous

Table 7.1. Kinetic parameter values used in the ADM1[a]

Parameter	Description	Value
k_{dis} (d^{-1})	Disintegration rate constant	0.5
$k_{hyd,ch}$(d^{-1})	Hydrolysis rate constant for carbohydrates	10
$k_{hyd,pro}$(d^{-1})	Hydrolysis rate constant for proteins	10
$k_{hyd,li}$(d^{-1})	Hydrolysis rate constant for lipids	10
k_{su} (d^{-1})	Sugar utilization rate constant	30
Kc_{su} (mg COD/L)	Half saturation constant for sugar utilization	500
k_{aa} (d^{-1})	Amino acid utilization rate constant	50
Kc_{aa} (mg COD/L)	Half saturation constant for amino acid	300
k_{fa} (d^{-1})	Fatty acid utilization rate constant	6
Kc_{fa} (mg COD/L)	Half saturation constant for fatty acid	400
k_{c4} (d^{-1})	C_4VFA utilization rate constant	20
Kc_{c4}(mg COD/L)	Half saturation constant for C4VFA utilization	200
k_{pro} (d^{-1})	Propionate utilization rate constant	13
Kc_{pro} (mg COD/L)	Half saturation constant for propionate	100
k_{ac} (d^{-1})	Acetate utilization rate constant	8
Kc_{ac} (mg COD/L)	Half saturation constant for acetate utilization	150
k_{h2} (d^{-1})	Hydrogen utilization rate constant	35
Kc_{h2} (mg COD/L)	Half saturation constant for hydrogen	7×10^{-3}
Y_{su} (mg COD/mg COD)	Yield coefficient of sugar degraders	0.1
Y_{aa} (mg COD/mg COD)	Yield coefficient of amino acid degraders	0.08
Y_{fa} (mg COD/mg COD)	Yield coefficient of fatty acid degraders	0.06
Y_{C4} (mg COD/mg COD)	Yield coefficient of C_4VFA degraders	0.06
Y_{pro} (mg COD/mg COD)	Yield coefficient of propionate degraders	0.04
Y_{ac} (mg COD/mg COD)	Yield coefficient of acetate degraders	0.05
Y_{h2} (mg COD/mg COD)	Yield coefficient of hydrogen degraders	0.06
$k_{dec, Xsu}$ (d^{-1})	Decay rate of sugar degraders	0.02
$k_{dec, Xaa}$ (d^{-1})	Decay rate of amino acid degraders	0.02
$k_{dec, Xfa}$ (d^{-1})	Decay rate of fatty acid degraders	0.02
$k_{dec, Xc4}$ (d^{-1})	Decay rate of C_4VFA degraders	0.02
$k_{dec, Xpro}$ (d^{-1})	Decay rate of propionate degraders	0.02
$k_{dec, Xac}$ (d^{-1})	Decay rate of acetate degraders	0.02
$k_{dec, Xh2}$ (d^{-1})	Decay rate of hydrogen degraders	0.02

[a] Batstone et al., 2002

oxide, nitric oxide, and nitrogen gas. Although the sulfide-free culture used in the present study had never been exposed to N-oxides, nitrate reduction occurred without any lag time in all nitrate-amended cultures. This result indicates that denitrifiers already existed in the culture and had survived by carrying out fermentation. Therefore, it was assumed that denitrification was carried out by a group of microorganisms, which was capable of fermenting in the absence of N-oxides (hereafter called fermentative denitrifiers). Butyrate/valerate (C_4VFA), propionate, acetate and H_2 were substrates used by the denitrifiers in the presence of N-oxides. In the absence of N-oxides, the fermentative denitrifiers carried out the fermentation of C_4VFA and propionate. Utilization of sugars, amino acids, and long-chain fatty acids (LCFAs) by the denitrifiers was neglected in the extended model because of the slower kinetics associated with the utilization of glucose and glutamic acid by denitrifiers as previously reported (Akunna et al., 1992). Because the denitrifiers could only use C_4VFA and propionate, initially 30% of the C_4VFA and propionate degraders were assumed to be the fermentative denitrifiers. The denitrifying group was assumed to be capable of reducing nitrate in a stepwise manner to NO_2^-, NO, N_2O and N_2. Although nitric oxide is not often detected as a denitrification intermediate, it was included in the model in order to simulate the complete biochemical pathway of denitrification. In addition, as discussed in Chapter 4, nitric oxide is the most inhibitory N-oxide among all denitrification intermediates and even trace levels may have a detrimental effect on fermentation and methanogenesis. All N-oxides served as electron acceptors, whereas nitrate and nitrite were assumed to be the nitrogen source for the denitrifiers.

The inhibitory effect of N-oxides on acetoclastic and hydrogenotrophic methanogens was simulated using non-competitive inhibition functions based on

previous reports (Klüber and Conrad, 1998a). However, the inhibitory effect of N-oxides on sugar, amino acid, LCFA, C_4VFA, and propionate degraders was neglected due to lack of such specific information. Based on published inhibitory effects of N-oxides on methanogens, the following inhibition constant (K_I) values were used in the extended model (in mg N/L): nitrate, 42; nitrite, 0.7; nitric oxide, 0.03; and nitrous oxide, 1.8 (Klüber and Conrad, 1998a). Possible inhibitory effects of N-oxides on denitrifiers were not included in the model because of lack of such specific information.

The electron donor utilization rates by the denitrifiers, calculated through stoichiometry and bioenergetics and modified via data calibration, are shown in Table 7.2. The utilization rates of N-oxides by the denitrifiers were correlated to electron donor utilization rates through stoichiometry because of the lack of information for all the electron donors and acceptors used in the model. A coefficient (f, mg N/mg substrate-COD; Table 7.2), which converts electron donor utilization rates to N-oxide utilization rates, was obtained from the mol ratio of N-oxide to electron donor from derived stoichiometric equations (see below). Yield coefficients were also calculated based on bioenergetics following procedures outlined by Rittmann and McCarty (2001). A single yield coefficient was used for each substrate (electron donor) using nitrate as the electron acceptor and values are shown in Table 7.2. Detailed stoichiometric equations are shown below for the reduction of nitrate using five electron donors: 1) valerate, 2) butyrate, 3) propionate, 4) acetate, and 5) H_2 (listed as electron donor/electron acceptor).

1-1) Valerate/nitrate reduction to nitrite:

$$CH_3(CH_2)_3COO^- + 7.8NO_3^- + 0.5NH_4^+$$

$$\rightarrow 1.9CO_2 + 0.5HCO_3^- + 7.8NO_2^- + 3.5H_2O + 0.5C_5H_7O_2N$$

1-2) Valerate/nitrite reduction to nitric oxide:

$$CH_3(CH_2)_3COO^- + 16.4NO_2^- + 16.4H^+ + 0.5NH_4^+$$

$$\rightarrow 2.1CO_2 + 0.5HCO_3^- + 16.4NO + 11.7H_2O + 0.5C_5H_7O_2N$$

1-3) Valerate/nitric oxide reduction to nitrous oxide:

$$CH_3(CH_2)_3COO^- + 12.8NO + 0.66NH_4^+$$

$$\rightarrow 1.36CO_2 + 0.34HCO_3^- + 6.4N_2O + 3.34H_2O + 0.66C_5H_7O_2N$$

1-4) Valerate/nitrous oxide reduction to nitrogen:

$$CH_3(CH_2)_3COO^- + 6.2N_2O + 0.7NH_4^+$$

$$\rightarrow 1.3CO_2 + 0.32HCO_3^- + 6.2N_2 + 3.32H_2O + 0.68C_5H_7O_2N$$

2-1) Butyrate/nitrate reduction to nitrite:

$$CH_3CH_2CH_2COO^- + 6NO_3^- + 0.4NH_4^+$$

$$\rightarrow 1.4CO_2 + 0.6HCO_3^- + 6NO_2^- + 2.6H_2O + 0.4C_5H_7O_2N$$

2-2) Butyrate/nitrite reduction to nitric oxide:

$$CH_3CH_2CH_2COO^- + 12.6NO_2^- + 12.6H^+ + 0.4NH_4^+$$

$$\rightarrow 1.5CO_2 + 0.6HCO_3^- + 12.6NO + 8.95H_2O + 0.4C_5H_7O_2N$$

2-3) Butyrate/nitric oxide reduction to nitrous oxide

$$CH_3CH_2CH_2COO^- + 9.84NO + 0.51NH_4^+$$

$$\rightarrow 0.97CO_2 + 0.49HCO_3^- + 4.92N_2O + 2.49H_2O + 0.51C_5H_7O_2N$$

2-4) Butyrate/nitrous oxide reduction to nitrogen:

$$CH_3CH_2CH_2COO^- + 4.78N_2O + 0.52NH_4^+$$
$$\rightarrow 0.91CO_2 + 0.48HCO_3^- + 4.78N_2 + 2.48H_2O + 0.52C_5H_7O_2N$$

3-1) Propionate/nitrate reduction to nitrite:

$$CH_3CH_2COO^- + 4.21NO_3^- + 0.28NH_4^+$$

$$\rightarrow 0.88CO_2 + 0.72HCO_3^- + 4.21NO_2^- + 1.72H_2O + 0.28C_5H_7O_2N$$

3-2) Propionate/nitrite reduction to nitric oxide:

$$CH_3CH_2COO^- + 8.85NO_2^- + 8.85H^+ + 0.26NH_4^+$$

$$\rightarrow 0.97CO_2 + 0.74HCO_3^- + 8.85NO + 6.17H_2O + 0.26C_5H_7O_2N$$

3-3) Propionate/nitrite reduction to nitrous oxide:

$$CH_3CH_2COO^- + 6.89NO + 0.36NH_4^+$$

$$\rightarrow 0.58CO_2 + 0.64HCO_3^- + 3.45N_2O + 1.64H_2O + 0.36C_5H_7O_2N$$

3-4) Propionate/nitrous oxide to nitrogen:

$$CH_3CH_2COO^- + 3.35N_2O + 0.37NH_4^+$$

$$\rightarrow 0.54CO_2 + 0.63HCO_3^- + 3.35N_2 + 1.63H_2O + 0.37C_5H_7O_2N$$

4-1) Acetate/nitrate reduction to nitrite:

$$CH_3COO^- + 1.84NO_3^- + 0.22NH_4^+$$
$$\rightarrow 0.14CO_2 + H^+ + 0.78HCO_3^- + 1.84NO_2^- + 0.78H_2O + 0.22C_5H_7O_2N$$

4-2) Acetate/nitrite reduction to nitric oxide:

$$CH_3COO^- + 5.08NO_2^- + 5.08H^+ + 0.1NH_4^+$$

$$\rightarrow 0.42CO_2 + 0.85HCO_3^- + 5.1NO + 3.4H_2O + 0.15C_5H_7O_2N$$

4-3) Acetate/nitric oxide reduction to nitrous oxide:

$$CH_3COO^- + 3.95NO + 0.2NH_4^+$$

$$\rightarrow 0.19CO_2 + 0.8HCO_3^- + 2N_2O + 0.8H_2O + 0.2C_5H_7O_2N$$

4-4) Acetate/nitrous oxide reduction to nitrogen:

$$CH_3COO^- + 1.92N_2O + 0.2NH_4^+$$

$$\rightarrow 0.17CO_2 + 0.79HCO_3^- + 1.9N_2 + 0.8H_2O + 0.21C_5H_7O_2N$$

5-1) Hydrogen/nitrate reduction to nitrite:

$$H_2 + 0.48NO_3^- + 0.21CO_2 + 0.05HCO_3^- + 0.05NH_4^+$$

$$\rightarrow 0.5NO_2^- + 0.9H_2O + 0.05C_5H_7O_2N$$

5-2) Hydrogen/nitrite reduction to nitric oxide:

$$H_2 + 1.01NO_2^- + 0.2CO_2 + 0.05HCO_3^- + 1.01H^+ + 0.05NH_4^+$$

$$\rightarrow NO + 1.5H_2O + 0.05C_5H_7O_2N$$

5-3) Hydrogen/nitric oxide reduction to nitrous oxide:

$$H_2 + 0.82NO + 0.24CO_2 + 0.06HCO_3^- + 0.06NH_4^+$$

$$\rightarrow 0.4N_2O + 0.9H_2O + 0.06C_5H_7O_2N$$

5-4) Hydrogen/nitrous oxide reduction to nitrogen:

$$H_2 + 0.4N_2O + 0.24CO_2 + 0.06HCO_3^- + 0.06NH_4^+$$

$$\rightarrow 0.4N_2 + 0.9H_2O + 0.06C_5H_7O_2N$$

Given the high growth rate of denitrifiers, their decay rate was taken as equal to 0.5 d^{-1}. The K_s values for the utilization of the four electron donors by the denitrifiers were chosen based on literature data and calibration to experimental data as shown in Table 7.2 (van Bodegom et al., 2001). The K_s value for nitrate is estimated from experimental data using Haldane equation (Chapter 6, Table 6.6). Although K_s values for nitrate have been estimated for all three substrates, propionate, acetate, and H_2/CO_2, a single value for nitrate has been used in the model. As a representative value, K_s value in the case of propionate utilization has been selected, which is 171 mg/L (Chapter 6, Table 6.6). The K_s values for the utilization of the other N-oxides were chosen based on literature values and calibration to experimental data, nitrite, 10; nitric oxide, 56; and nitrous oxide, 28 (Betlach and Tiedje, 1981; Zumft, 1997). In addition to methane, hydrogen and carbon dioxide gases described in the ADM1, nitric oxide, nitrous oxide, and nitrogen were also introduced as gaseous

Table 7.2. Kinetic parameter values used in the extended ADM1[a]

Electron Donor	Electron Acceptor	k (mg substrate COD/mg biomass-COD · d)	Y (mg biomass-COD/ mg substrate-COD)	f (mg N/mg substrate-COD)	K_C (mg COD/L)
C_4VFA	NO_3^-	3	0.32	0.40	100
	NO_2^-	1.5		0.87	
	NO	18.7		0.53	
	N_2O	20.5		0.48	
Propionate	NO_3^-	0.265	0.32	0.40	500
	NO_2^-	1.45		0.87	500
	NO	18.7		0.53	250
	N_2O	20.5		0.49	250
Acetate	NO_3^-	1.34	0.32	0.40	500
	NO_2^-	1.45		0.88	500
	NO	18.4		0.53	250
	N_2O	20.1		0.49	250
Hydrogen (H_2)	NO_3^-	1.35	0.12	0.71	1.6×10^{-3}
	NO_2^-	0.18		1.45	
	NO	8.1		1.23	
	N_2O	8.3		1.19	

[a] See text

species in the extended model with Henry's law constant values of 1.82, 24.6, and 0.631 mol/m^3-bar (Stumm and Morgan, 1996).

The process equations for soluble substrates (for i = 4-8) in the ADM1 were modified to include denitrification (Equation 1):

$$\frac{dS_i}{dt} = \frac{q}{V_{liq}}(S_{i,in} - S_i) - \sum_{j=5-7} \rho_j \nu_{i,j} - \sum_{j=8-10} (\rho_j + \rho_{j,nox}) \nu_{i,j} - \sum_{j=11-12} \rho_j \nu_{i,j} I_{nox} - \rho_{i,nox} \quad (1)$$

where, S_i is the concentration of component i (electron donor)(mg COD/L), $\nu_{i,j}$ is the rate coefficient for component i on process j (dimensionless), ρ_j is the rate for process j (mg COD/L · d), $\rho_{j,nox}$ is the rate for process j during fermentation by denitrifiers (mg COD/L · d), and $\rho_{i,nox}$ is the rate of uptake of soluble substrate i during denitrification (mg COD/L · d). The first part of equation 1 is the substrate mass balance for a CSTR. The second part is the process equations for soluble substrates, obtained from the ADM1 matrix. The third part of equation 1 represents the utilization of C_4VFA and propionate by fermentative denitrifiers in the absence of N-oxides. The fourth part of equation 1 denotes the process equations for acetate and H_2 utilization by methanogens multiplied by an N-oxide inhibition factor (I_{nox}) in order to account for the inhibitory effect of N-oxide on methanogenesis. The fifth part of equation 1 represents the uptake of soluble substrates by denitrifiers in the presence of N-oxides. The detailed equations can be found in the matrix of the extended model (Table 7.3). The general rate equation for the utilization of C_4VFA and propionate by fermentative denitrifiers in the absence of N-oxides is expressed as follows:

$$\rho_{j,nox} = k_{m,j} \frac{S_i}{K_s + S_i} \left[\frac{K_{s,no3}}{K_{s,no3} + S_{no3}} \frac{K_{s,no2}}{K_{s,no2} + S_{no2}} \frac{K_{s,no}}{K_{s,no} + S_{no}} \frac{K_{s,n2o}}{K_{s,n2o} + S_{n2o}} \right] \left[\frac{S_i}{S_{va} + S_{bu} + S_{pro}} X_{f,nox} \right] \quad (2)$$

where, $k_{m,j}$ is the rate constant for the fermentation of process j (mg substrate COD/mg biomass-COD · d), and $X_{f,nox}$ is the fermentative denitrifying population (mg biomass-

Table 7.3. Matrix of the extended ADM1 related to denitrification processes

Component		Process Rates, $\rho_{i,n}$				Overall Process rate
	n → i↓	NO_3^-	NO_2^-	NO	N_2O	$\rho_{i,nox}$
Valerate S_{va}	4	$k_{no3,va}\frac{S_{va}}{K_{no3,va}+S_{va}}X_{f,nox}\frac{S_{no3}}{K_{no3}+S_{no3}}$	$k_{no2,va}\frac{S_{va}}{K_{no2,va}+S_{va}}X_{f,nox}\frac{S_{no2}}{K_{no2}+S_{no2}}$	$k_{no,va}\frac{S_{va}}{K_{no,va}+S_{va}}X_{f,nox}\frac{S_{no}}{K_{no}+S_{no}}$	$k_{n2o,va}\frac{S_{va}}{K_{n2o,va}+S_{va}}X_{f,nox}\frac{S_{n2o}}{K_{n2o}+S_{n2o}}$	$\sum_{n=no3-n2o}\rho_{4,n}$
Butyrate S_{bu}	5	$k_{no3,bu}\frac{S_{bu}}{K_{no3,bu}+S_{bu}}X_{f,nox}\frac{S_{no3}}{K_{no3}+S_{no3}}$	$k_{no2,bu}\frac{S_{bu}}{K_{no2,bu}+S_{bu}}X_{f,nox}\frac{S_{no2}}{K_{no2}+S_{no2}}$	$k_{no,bu}\frac{S_{bu}}{K_{no,bu}+S_{bu}}X_{f,nox}\frac{S_{no}}{K_{no}+S_{no}}$	$k_{n2o,bu}\frac{S_{bu}}{K_{n2o,bu}+S_{bu}}X_{f,nox}\frac{S_{n2o}}{K_{n2o}+S_{n2o}}$	$\sum_{n=no3-n2o}\rho_{5,n}$
Propionate S_{pro}	6	$k_{no3,pro}\frac{S_{pro}}{K_{no3,pro}+S_{pro}}X_{f,nox}\frac{S_{no3}}{K_{no3}+S_{no3}}$	$k_{no2,pro}\frac{S_{pro}}{K_{no2,pro}+S_{pro}}X_{f,nox}\frac{S_{no2}}{K_{no2}+S_{no2}}$	$k_{no,pro}\frac{S_{pro}}{K_{no,pro}+S_{pro}}X_{f,nox}\frac{S_{no}}{K_{no}+S_{no}}$	$k_{n2o,pro}\frac{S_{pro}}{K_{n2o,pro}+S_{pro}}X_{f,nox}\frac{S_{n2o}}{K_{n2o}+S_{n2o}}$	$\sum_{n=no3-n2o}\rho_{6,n}$
Acetate S_{ac}	7	$k_{no3,ac}\frac{S_{ac}}{K_{no3,ac}+S_{ac}}X_{f,nox}\frac{S_{no3}}{K_{no3}+S_{no3}}$	$k_{no2,ac}\frac{S_{ac}}{K_{no2,ac}+S_{ac}}X_{f,nox}\frac{S_{no2}}{K_{no2}+S_{no2}}$	$k_{no,ac}\frac{S_{ac}}{K_{no,ac}+S_{ac}}X_{f,nox}\frac{S_{no}}{K_{no}+S_{no}}$	$k_{n2o,ac}\frac{S_{ac}}{K_{n2o,ac}+S_{ac}}X_{f,nox}\frac{S_{n2o}}{K_{n2o}+S_{n2o}}$	$\sum_{n=no3-n2o}\rho_{7,n}$
Hydrogen S_{h2}	8	$k_{no3,h2}\frac{S_{h2}}{K_{no3,h2}+S_{h2}}X_{f,nox}\frac{S_{no3}}{K_{no3}+S_{no3}}$	$k_{no2,h2}\frac{S_{h2}}{K_{no2,h2}+S_{h2}}X_{f,nox}\frac{S_{no2}}{K_{no2}+S_{no2}}$	$k_{no,h2}\frac{S_{h2}}{K_{no,h2}+S_{h2}}X_{f,nox}\frac{S_{no}}{K_{no}+S_{no}}$	$k_{n2o,h2}\frac{S_{h2}}{K_{n2o,h2}+S_{h2}}X_{f,nox}\frac{S_{n2o}}{K_{n2o}+S_{n2o}}$	$\sum_{n=no3-n2o}\rho_{8,n}$
Nitrate S_{no3}	25	$-\sum_{i=4-8}f_i\rho_{i,no3}$				$\sum_{n=no3-n2o}\rho_{25,n}$
Nitrite S_{no2}	26	$\sum_{i=4-8}f_i\rho_{i,no3}$	$-\sum_{i=4-8}f_i\rho_{i,no2}$			$\sum_{n=no3-n2o}\rho_{26,n}$
Nitric oxide S_{no}	27		$\sum_{i=4-8}f_i\rho_{i,no2}$	$-\sum_{i=4-8}f_i\rho_{i,no}$		$\sum_{n=no3-n2o}\rho_{27,n}$
Nitrous oxide S_{n2o}	28			$0.5\sum_{i=4-8}f_i\rho_{i,no}$	$-\sum_{i=4-8}f_i\rho_{i,n2o}$	$\sum_{n=no3-n2o}\rho_{28,n}$
Nitrogen S_{n2}	29				$\sum_{i=4-8}f_i\rho_{i,n2o}$	$\sum_{n=no3-n2o}\rho_{29,n}$
NOx degraders $X_{f,nox}$	30	$\sum_{i=4-8}Y_{i,Xf,nox}\rho_{i,no3}$	$\sum_{i=4-8}Y_{i,Xf,nox}\rho_{i,no2}$	$\sum_{i=4-8}Y_{i,Xf,nox}\rho_{i,no}$	$\sum_{i=4-8}Y_{i,Xf,nox}\rho_{i,n2o}$	$\sum_{n=no3-n2o}\rho_{30,n}+\sum_{j=8-10}(Y_{c4}+Y_{pro})\rho_{j,nox}$

f_i , stoichiometric factor for converting the rate of substrate (electron donor) utilization to the rate of N-oxide reduction (kg N/kg biomass-COD)(see Table 1); n, N-oxides utilized as electron acceptors in denitrification.

COD/L). The first part of equation 2 represents the substrate utilization with the kinetic rates suggested in the ADM1 report. The second part of the equation depicts the C_4VFA and propionate utilization (fermentation or denitrification) that the denitrifiers will carry out depending on the N-oxide concentration. The third part of equation 2 determines the concentration of denitrifiers associated with the utilization of valerate, butyrate, or propionate, depending on the concentration of each substrate. The general rate equation for the utilization of N-oxides (for i = 25-29) is as follows:

$$\frac{dS_{i,nox}}{dt} = \frac{q}{V_{liq}}(S_{i,nox-in} - S_{i,nox}) + \rho_{i,nox} \quad (3)$$

where, $S_{i,nox}$ is the concentration of N-oxide i (mg N/L) and $\rho_{i,nox}$ is the rate of production/consumption of N-compound i as a result of denitrification (mg N/L · d).

The pH value for denitrification varied from 5 to 9 in the extended ADM1. The reason for selecting such a high boundary for pH was due to unstable CO_2 and thus pH calculations in the ADM1, which resulted in the inhibition of denitrification at neutral pH values (Smith and Chen, 2006). In all assays the culture pH value never increased to inhibitory values and varied between 7.01 and 8.06. Therefore, such simplification in the model is reasonable and justifiable.

It should be noted that such a complex model may not be necessary to describe continuous-flow systems in which nitrate reduction may not result in the accumulation of N-oxides and where the effect of nitrate reduction may be accounted for by a proportional decrease in the biodegradable COD available for fermentation and methane production. However, the model complexity as presented here is necessary for either batch systems or continuous-flow systems experiencing shock loads of nitrate.

7.3. Model Implementation and Simulation

7.3.1. Model Implementation – Batch Systems

A batch assay to test the effect of nitrate on a mixed fermentative/methanogenic culture was performed using the sulfide-free enriched culture as described in Chapter 4. A mixture of dextrin/peptone served as the carbon and energy source at an initial concentration of 1000 and 500 mg COD/L, respectively. A series of subcultures were setup with an initial nitrate concentration of 0 (control culture), 10, 75, and 150 mg NO_3^--N/L. The initial biomass concentration of each microbial group in the model was assigned based on the total biomass concentration used in the laboratory assay, multiplied by the ratio of the observed yield coefficient of each microbial group to the overall observed yield coefficient. Initial total active biomass amount of the mixed methanogenic stock culture was approximately 2200 mg COD/L. During the setup of the experiment the seed was diluted with the culture media and nitrate solution to a final concentration of 1370 mg COD/L. In order to estimate the initial biomass amount for each biological group, the ratio of their observed yield coefficient (Y_{obs}) to overall Y_{obs} was calculated and multiplied by the initial total active biomass amount (Table 7.2). The Y_{obs} values were calculated from the yield coefficients and decay rate (0.02 d^{-1}) suggested in the ADM1 (Table 7.1). Considering that the stock methanogenic culture was not contacted with nitrate before, the initial amount of denitrifiers was assumed to be very low. The initial biomass concentrations used in the model were as follows (in mg COD/L): 303 sugar degraders, 242 amino acid degraders, 182 fatty acid degraders, 182 C_{4+} VFA degraders, 121 propionate degraders, 152 acetate degraders, 182 H_2 degraders, and 100 denitrifiers. Other initial parameters were assumed the same as in the ADM1.

Model simulation results along with experimental data for the control culture (without nitrate addition) are shown in Figure 7.1. The model modifications relative to the disintegration and hydrolysis rate values of the feed and biomass enabled the model to accurately simulate the initial fast rate of methane production (Figure 7.1A), as well as the production and consumption of VFAs (Figure 7.1B). Significantly higher acetate and propionate production was predicted when the disintegration and hydrolysis rate values suggested in the ADM1 report were used (Figure 7.1C).

The experimental results showed that addition of nitrate resulted in immediate suppression of methanogenesis in the 75 and 150 mg N/L nitrate-amended cultures. The initial methane production rate in the 10 mg N/L nitrate-amended culture was lower compared to the control culture (Figure 7.2A). Both the initial suppression of methanogenesis and the methane production after the resumption of methanogenesis, as well as the rate and extent of N_2 production were well simulated by the model (Figures 7.2A and 7.3C). The lower extent of methane production in the nitrate-amended cultures, compared to the control culture, is the result of denitrification (i.e., electron equivalents used for nitrate reduction).

In all nitrate-amended cultures, similar trends were obtained in terms of levels of acetate, propionate, nitrate, and nitrite. Complete reduction of nitrate and nitrite occurred in less than 2 days (Figures 7.2B and 7.2C), followed by the resumption of methanogenesis (Figure 7.2A) in all the nitrate-amended cultures. Although the N_2O and NO levels could not be monitored due to analytical limitations, model simulations showed that complete N_2O and NO reduction also occurred within 2 days in all nitrate-amended cultures (Figures 7.3A and 7.3B) suggesting that while nitrate, nitrite, NO, and N_2O were present, methanogenesis did not take place. Although the higher order carbon sources could not be monitored during the

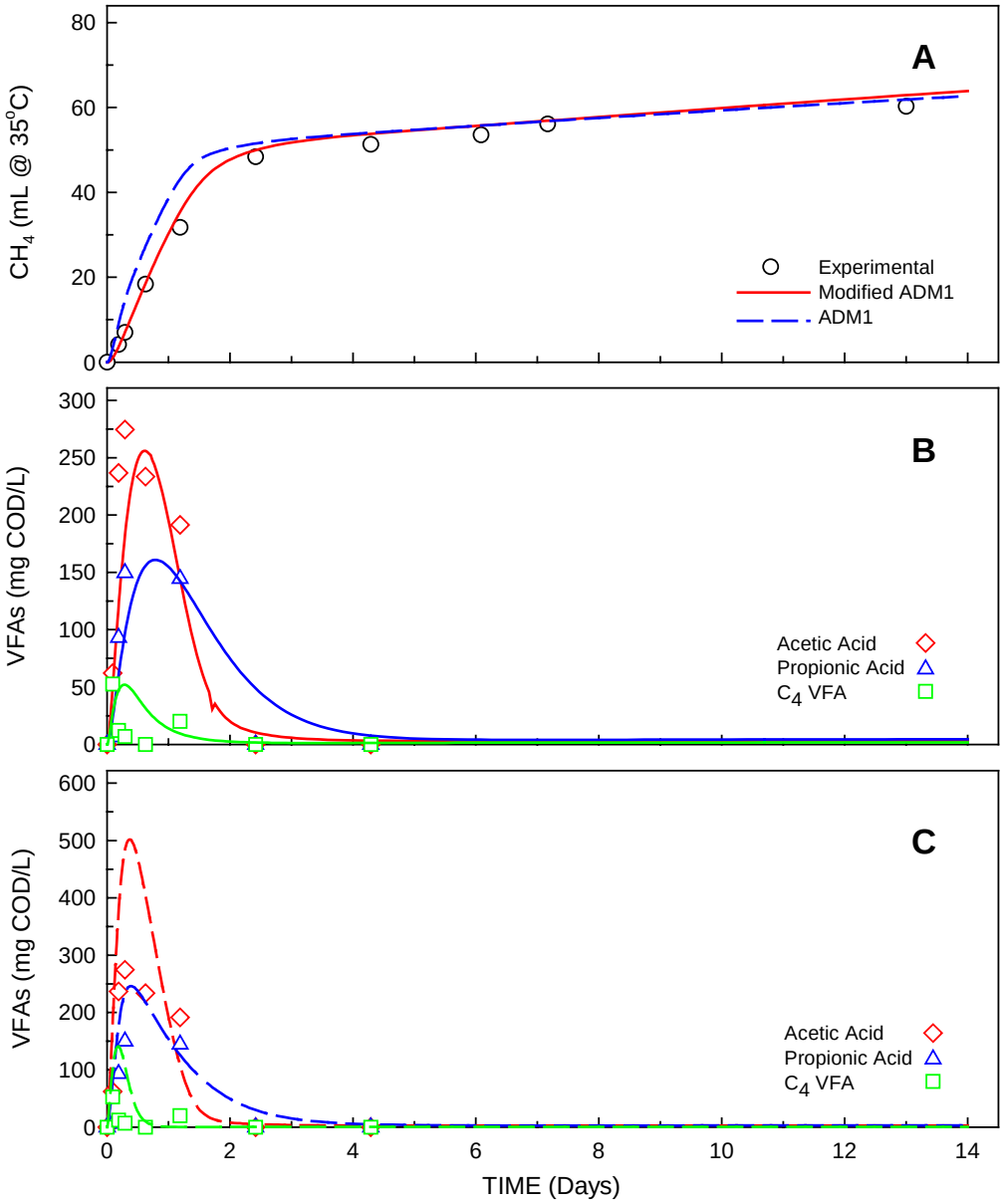

Figure 7.1. Experimental data and model simulation of (A) methane production for both modified and unmodified disintegration and hydrolysis rates, (B) VFA production and consumption for modified, and (C) unmodified disintegration and hydrolysis rates in the control culture (without any nitrate addition). Colored figure.

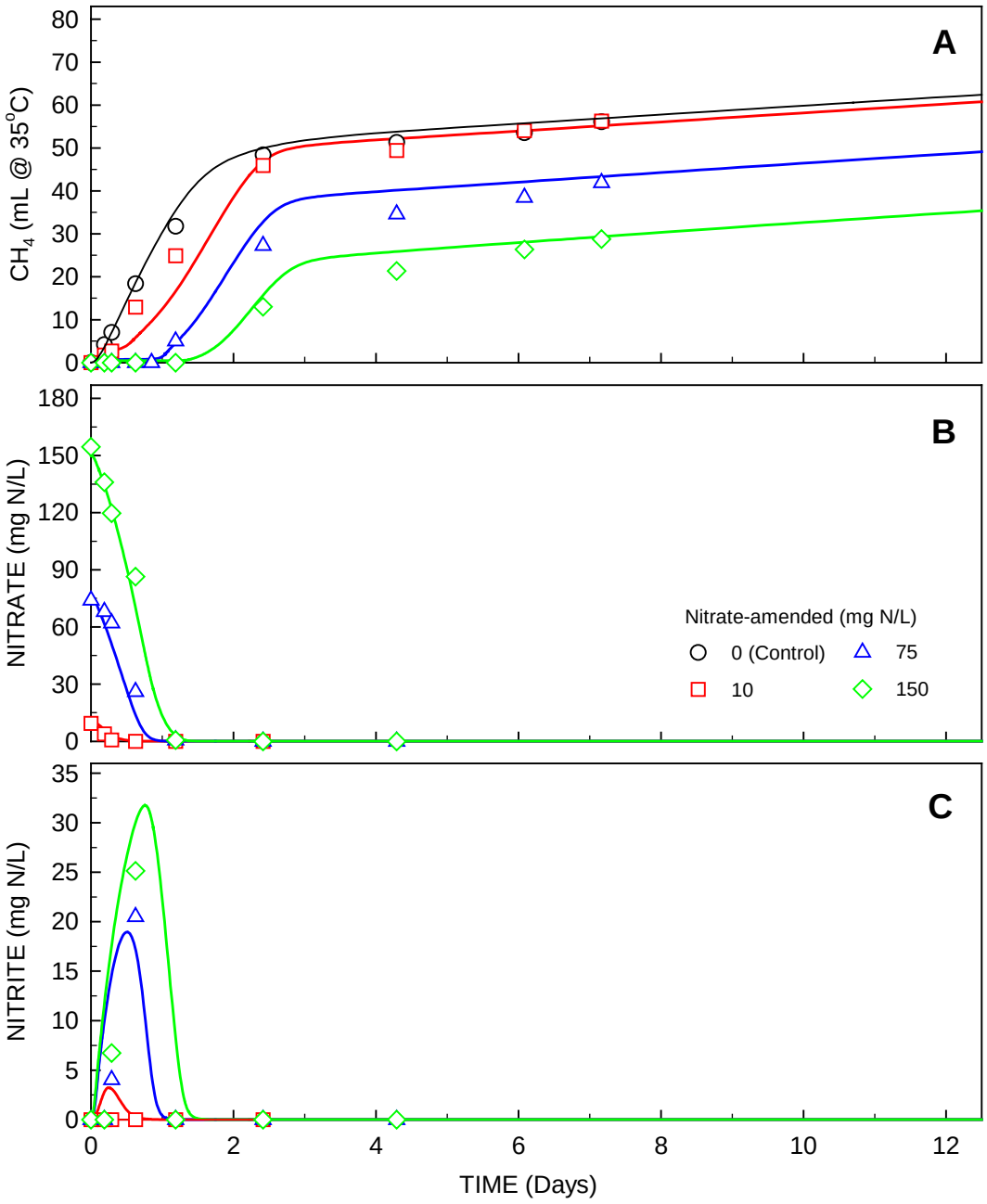

Figure 7.2. Experimental data and model simulation for the 0 (control), 10, 75, 150 mg N/L nitrate-amended cultures (A) methane production, (B) nitrate reduction, (C) nitrite production and reduction profiles. Colored figure.

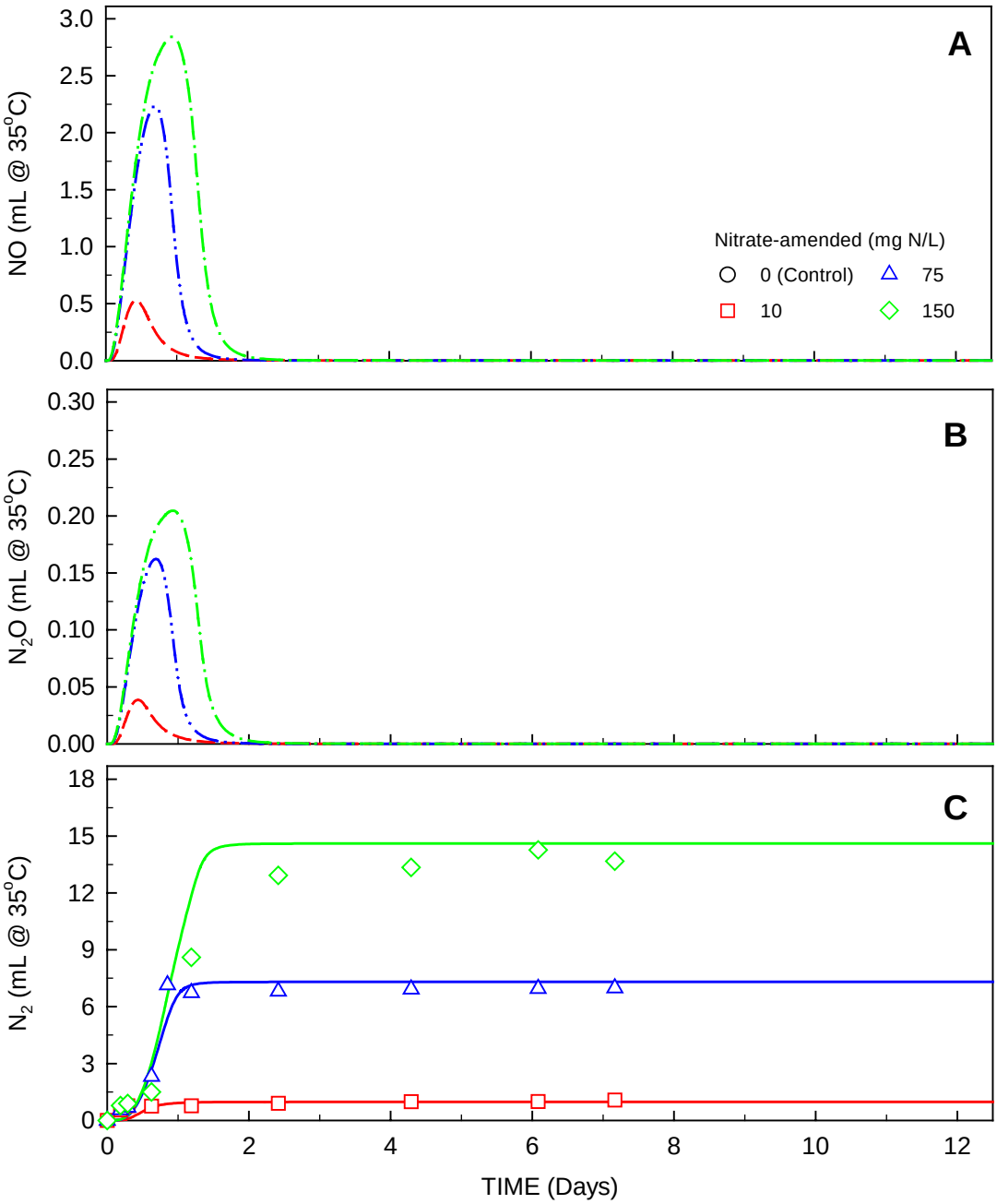

Figure 7.3. Experimental data and/or model simulation for the 0 (control), 10, 75, and 150 mg N/L nitrate-amended cultures (A) nitric oxide, (B) nitrous oxide, and (C) nitrogen gas production profiles. Colored figure.

experiment, they were simulated using the model. The results of these simulations are presented in Figures 7.4, 7.5, and 7.6. Carbohydrates and protein were supplied to the culture by the addition of dextrin and peptone, respectively. Therefore, carbohydrates and proteins were utilized and converted to sugars and amino acids. Utilization of carbohydrates, proteins, sugars, and amino acids were not very different among the 0 (control), 10, 75, and 150 mg N/L nitrate-amended cultures (Figures 7.4A, 7.4B, 7.4C, and 7.5A). The nitrate reducers did not utilize high order carbons and the utilization of these substrates was not affected by the presence of N-oxides (i.e., N-oxides were not inhibitory); therefore, similar profiles even in the presence of different amounts of N-oxides were expected. Although lipids were not added to the system, fatty acid production was observed as a result of biomass decay (Figure 7.5B). Differences in the fatty acid levels were observed among the cultures amended with different initial nitrate concentrations, which was due to higher biomass growth and therefore decay in the cultures amended with high nitrate concentrations (e.g., 75 and 150 mg N/L nitrate-amended cultures) (Figure 7.5 B). Higher accumulation of valerate and butyrate was observed in the nitrate-amended cultures compared to the control culture (Figures 7.5C and 7.6A). Production and consumption of acetate and propionate were well simulated by the model (Figures 7.6B and 7.6C). At the time when complete nitrate and nitrite reduction was observed (Figures 7.2B and 7.2C), the model simulation showed a temporary, sudden decrease in the rate of acetate utilization (Figure 7.6C), which is attributed both to the cessation of acetate consumption by the denitrifiers and an increase in the fermentation rate of higher order VFAs due to the increase in the population size of fermenting species after all N-oxides were consumed. A similar profile was also observed for the H_2 production and consumption (Figure 7.6D). Biomass growth profiles were similar for the sugar,

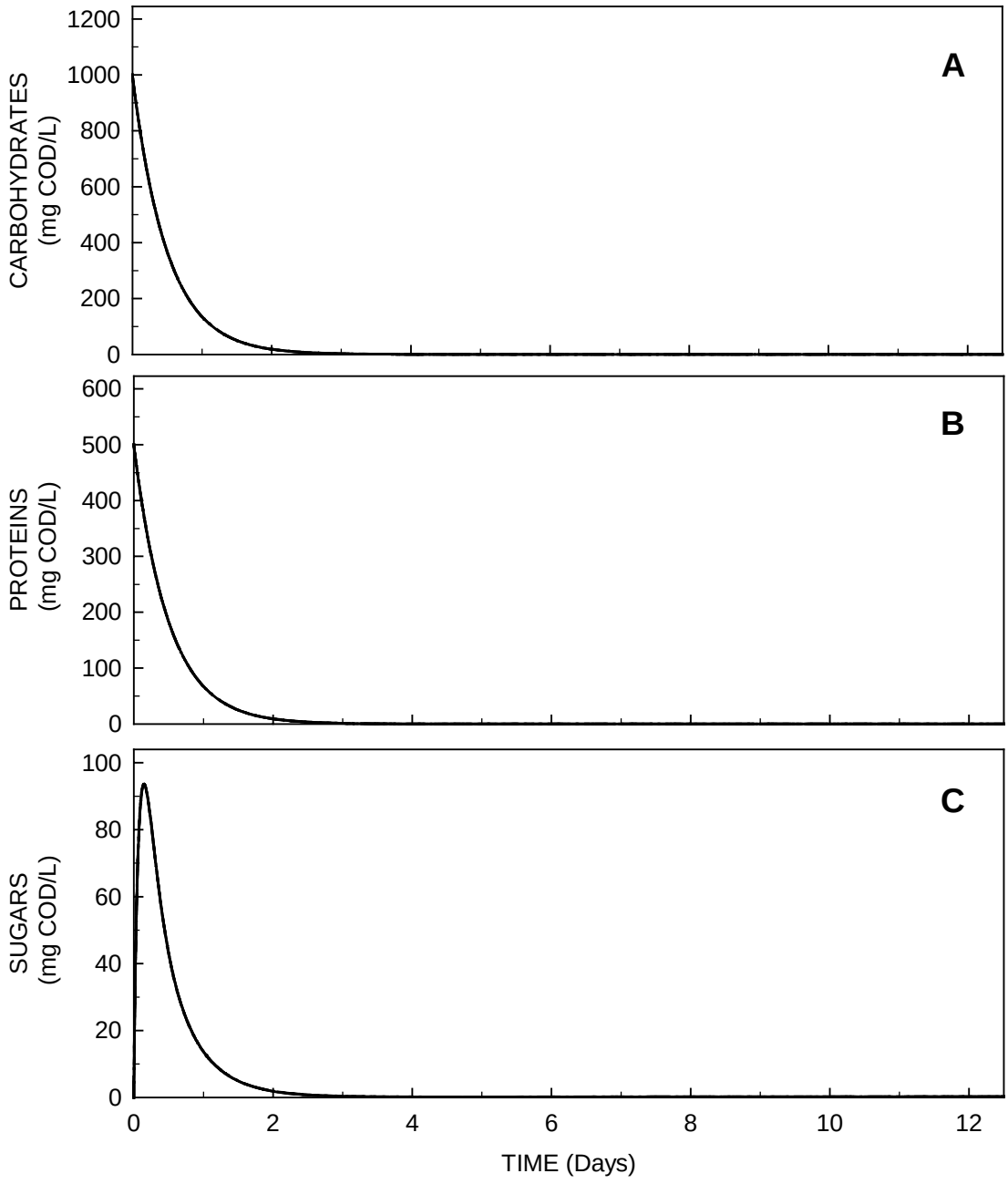

Figure 7.4. Model prediction for the (A) carbohydrate, (B) protein, and (C) sugar utilization profiles for 0, 10, 75, and 150 mg N/L nitrate-amended cultures (Model prediction shows the same profiles for all three nitrate-amended cultures).

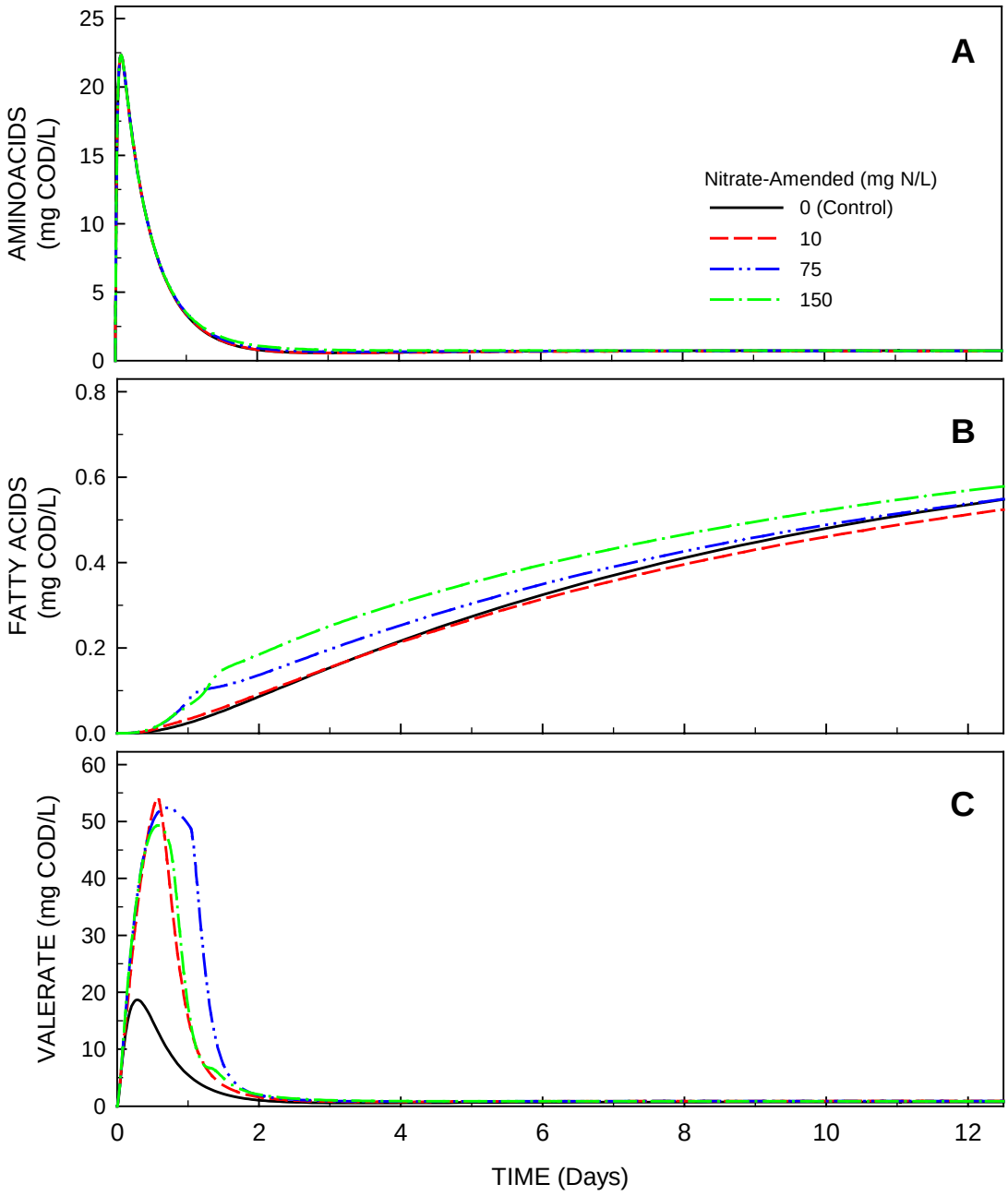

Figure 7.5. Model prediction for the (A) amino acids, (B) fatty acids, and (C) valerate utilization profiles for the 0, 10, 75, and 150 mg N/L nitrate-amended cultures. Colored figure.

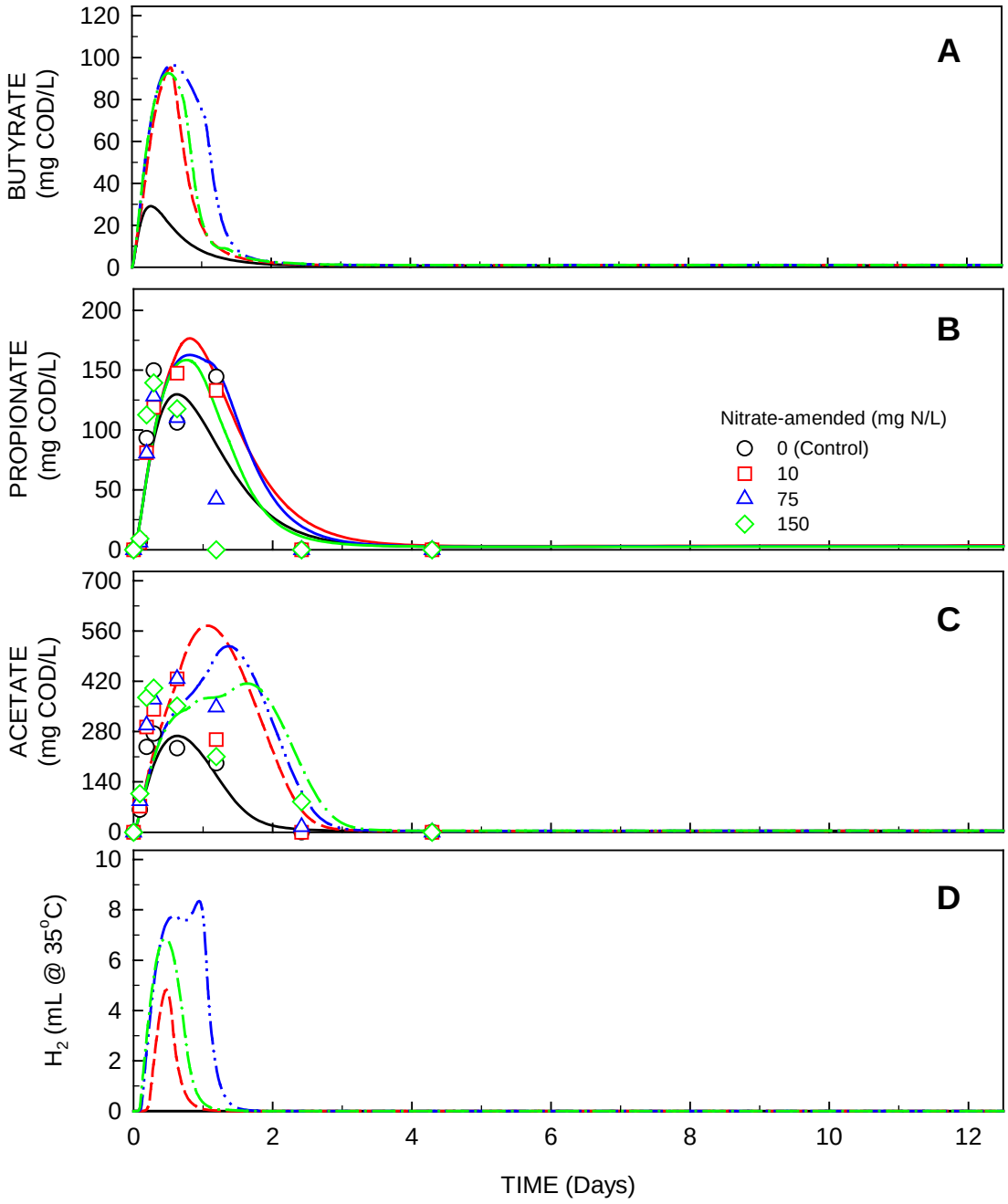

Figure 7.6. Experimental data and/or model prediction for the (A) butyrate, (B) propionate, (C) acetate, and (D) hydrogen production and utilization profiles for the 0, 10, 75, and 150 mg N/L nitrate-amended cultures. Colored figure.

amino acid, and fatty acid degraders in the cultures amended with different initial nitrate concentrations due to the same reasons explained above for sugar, amino acid, and fatty acid utilization (Figures 7.7A, 7.7B, and 7.7C). However, the levels of C_4VFA and propionate degraders were lower in the nitrate-amended cultures due to the switch of a fraction of C_4VFA and propionate degraders to nitrate utilization in the presence of nitrate (Figures 7.7D and 7.8A). Growth was not observed for the acetoclastic methanogens as long as N-oxides were present in the system, which was due to the inhibitory effects of N-oxides on methanogens (Figure 7.8B). Although less pronounced, the growth of hydrogenotrophic methanogens was also suppressed as long as N-oxides remained in the system (Figure 7.8C).

The significance of N-oxide inhibition relative to the observed suppression of methanogenesis in the nitrate-amended cultures was investigated using model simulation as follows. The effect of N-oxide inhibition was tested by removing the inhibition functions, which is tantamount of assuming that the interactions between methanogenesis and denitrification are based purely on stoichiometry and kinetics. Removal of the inhibition functions resulted in immediate methane production (i.e., no suppression), but also led to incomplete nitrate removal and accumulation of nitrite due to the inability of denitrifiers to effectively compete with the fermenting and methanogenic species for electron equivalents (Figures 7.9A and 7.9B). These results demonstrate that the effect of nitrate reduction on a fermenting/methanogenic system cannot be explained based purely on stoichiometric and kinetic grounds. Inhibition by intermediate N-oxides plays a very important role on the overall process dynamics.

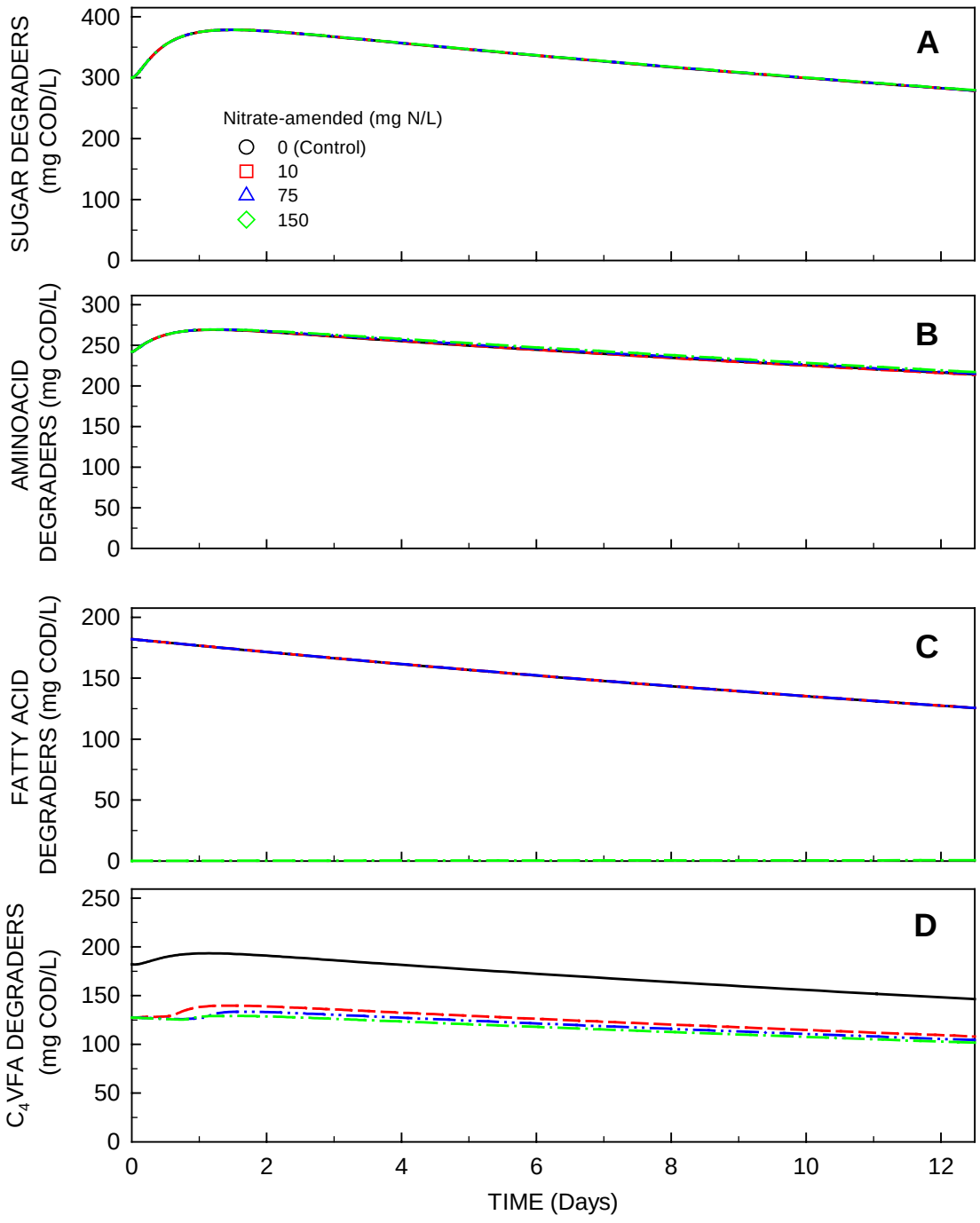

Figure 7.7. Model predictions for the growth of (A) sugar, (B) amino acid, (C) fatty acid, and (D) C_4VFA degraders in the 0, 10, 75, and 150 mg N/L nitrate-amended cultures. Colored figure.

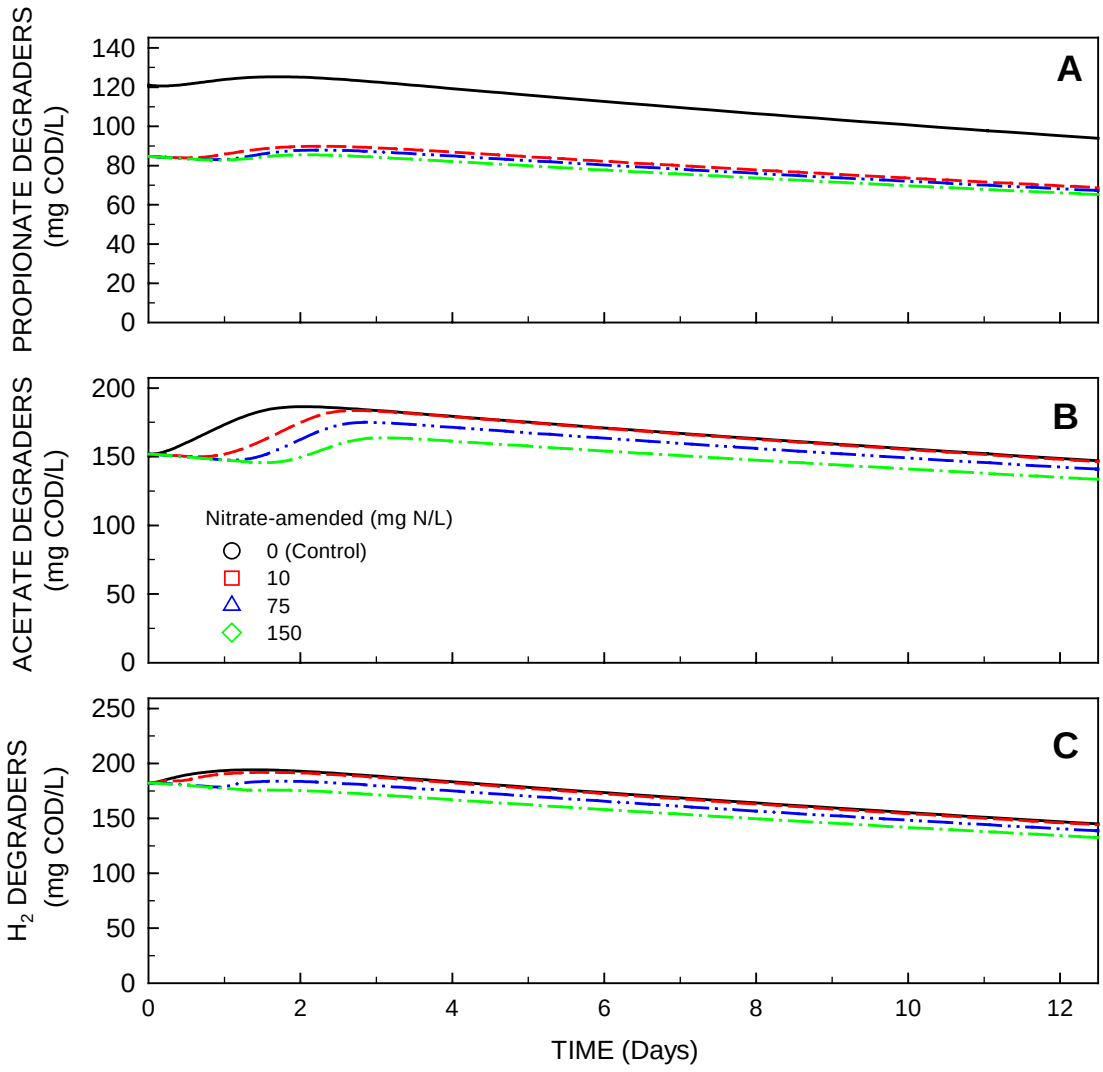

Figure 7.8. Model predictions for the growth of (A) propionate, (B) acetate, and (C) hydrogen degraders in the 0, 10, 75, and 150 mg N/L nitrate-amended cultures. Colored figure.

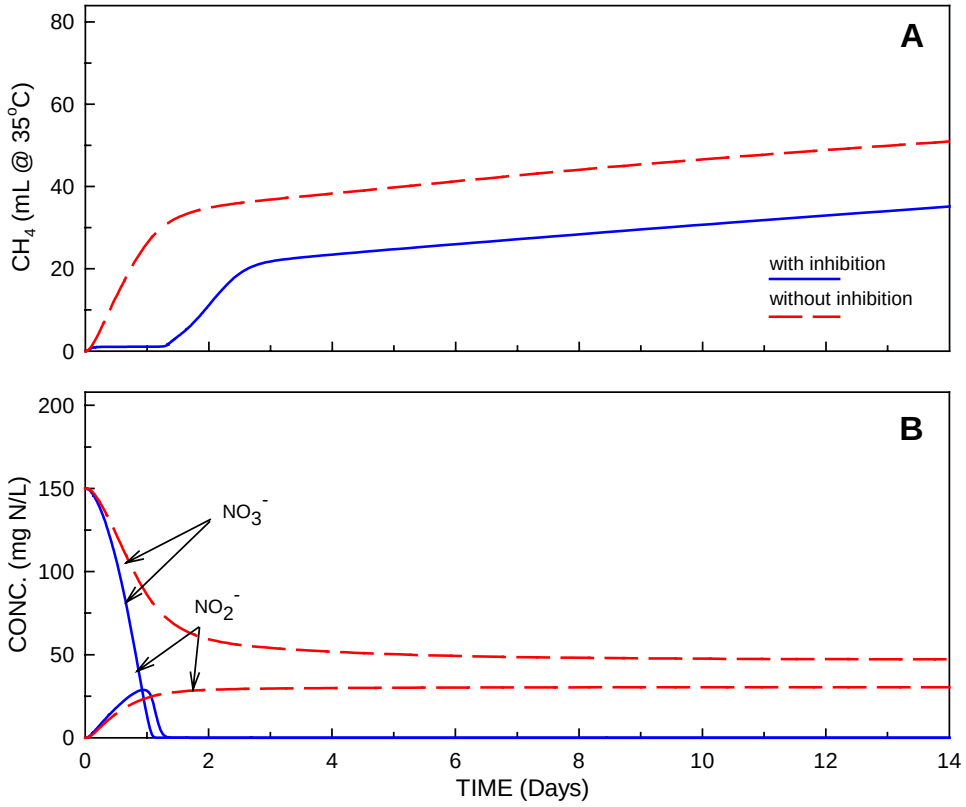

Figure 7.9. Model simulations showing the effect of removing the N-oxides inhibition functions for methanogenic species on (A) methane production and (B) nitrate/nitrite reduction (Initial nitrate concentration equal to 150 mg N/L). Colored figure.

7.3.2. Sensitivity analysis – Batch System

A sensitivity analysis was performed in order to determine the effect of k' and K_c parameter values for nitrate, nitrite, nitric oxide, and nitrous oxide reduction on the nitrate reduction rate when valerate, butyrate, propionate, acetate, and hydrogen were the electron donors. The results of the sensitivity analysis are given in Table 7.4. Changes in the k_{h2no3} and K_c values resulted in the highest effect on nitrate reduction rates regardless of the type of substrate used. For all the k_{no3} values, 10% increase in the parameter value resulted in a positive increase in the nitrate reduction rate, whereas 10% decrease in the parameter value caused nitrate reduction rates to decrease (Table 7.4). The observed highest impact with the change of k_{h2no3} value could be the result of the high rate of nitrate reduction in the presence of hydrogen as an electron donor. The parameters related to nitrite, nitric oxide, and nitrous oxide utilization had equal importance and minimal impact on nitrate reduction. The nitrate reduction was affected mostly by the changes in parameters related to nitrate reduction.

7.3.3. Effect of the denitrifying biomass size – Batch Systems

In order to determine the effect of the amount of denitrifying biomass on the fermentation/methanogenesis and nitrate reduction, the fraction of C_4VFA and propionate degraders, which are capable of denitrifying, was varied and simulated using the model. Denitrifiers were assumed to be 30% of the C_4VFA and propionate degraders in the baseline model. This fraction was changed to 1, 2, 5, 10, 20, 40, and 50% and model simulations are shown in Figures 7.10 through 7.13. The fastest and the slowest methane production rate was observed when the fraction of denitrifiers

Table 7.4. Results of the sensitivity analysis of model parameters

Parameter	Base-line parameter value	Percent change in nitrate reduction rate with	
		10 % increase in parameter value	10 % decrease in parameter value
$k_{no3,val}$[a]	3	0.6	-0.5
$k_{no3,but}$[a]	3	1.3	-1.3
$k_{no3,pro}$[a]	0.265	0.1	-0.1
$k_{no3,ace}$[a]	1.34	0.7	-0.8
$k_{no3,h2}$[a]	1.34	4.9	-5.3
K_{no3}[b]	171	-4.6	5.1
$k_{no2,val}$[a]	1.45	0.1	-0.1
$k_{no2,but}$[a]	1.45	0	0
$k_{no2,pro}$[a]	1.45	0.1	-0.1
$k_{no2,ace}$[a]	1.45	0.2	0
$k_{no2,h2}$[a]	0.18	0.1	0.1
K_{no2}[b]	10		
$k_{no,val}$[a]	18.7	0.05	0.06
$k_{no,but}$[a]	18.7	0.03	0.08
$k_{no,pro}$[a]	18.7	0.09	0.02
$k_{no,ace}$[a]	18.4	0.02	0
$k_{no,h2}$[a]	8.1	0.03	0.01
K_{no}	56	0.01	0.01
$k_{n2o,val}$[a]	20.5	0.05	0.02
$k_{n2o,but}$[a]	20.5	0	0.03
$k_{n2o,pro}$[a]	20.5	0.02	0.05
$k_{n2o,ace}$[a]	20.1	0.03	0.02
$k_{n2o,h2}$[a]	8.3	0.03	0
K_{n2o}[b]	28	0.05	0

[a] In the units of mg substrate COD/mg biomass-COD · d
[b] In the units of mg COD/L

was 50 and 1%, respectively (Figure 7.10A). When the fraction of denitrifiers decreased below 20%, a significant decrease in the nitrate reduction rate was observed, which contributed to a longer cessation of methanogenesis, as well as a lower rate of methane production upon recovery (Figures 7.10A and 7.10B). As the fraction of denitrifiers decreased below 20%, accumulation of N-oxides was more pronounced, which resulted in longer inhibition of methanogenesis (Figures 7.10B, 7.10C, 7.11A, and 7.11B). In addition, as the denitrifiers fraction became lower, the lag period before the onset of nitrate reduction increased which resulted in lower nitrate reduction rates at the beginning of the incubation (Figure 7.10B). Accumulation of denitrification intermediates also resulted in slower nitrogen gas production (Figure 7.11C). The decrease in the biomass concentration also affected the rate of both propionate and acetate utilization. As the denitrifying biomass fraction decreased, propionate accumulation increased as a result of slow propionate utilization by the denitrifiers (Figure 7.12A). In addition, as a result of inhibition of methanogenesis, the acetate utilization rate was significantly decreased at a low initial denitrifying population size (Figure 7.12B). However, the lowest accumulation of H_2 was observed at the lowest initial denitrifying biomass concentration, which was presumably due to the slow utilization of propionate, and thus lower accumulation of H_2 resulting from the fermentation of propionate (Figure 7.12C). The initial concentration as well as the denitrifying biomass concentration over time are shown in Figure 7.13A. The growth of acetate utilizers was affected as a result of inhibition by accumulated N-oxides brought about by the small initial denitrifying population (Figure 7.13B). The hydrogenotrophic methanogenic population was also affected by the accumulation of N-oxides, albeit this effect was less noticeable due to the lower inhibitory effect of N-oxides on H_2 utilizers (Figure 7.13C).

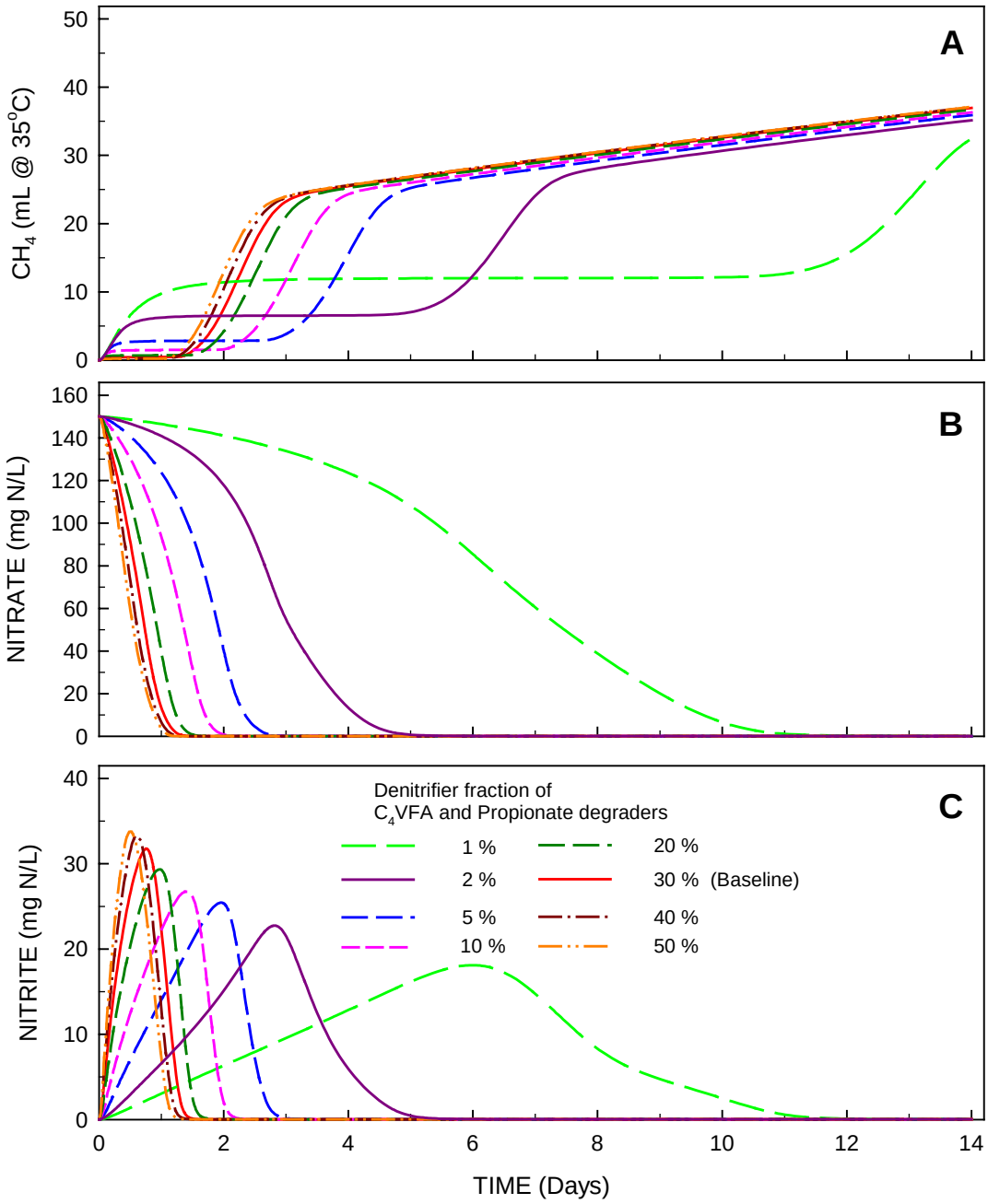

Figure 7.10. Effect of the initial denitrifying population size on (A) methane production, (B) nitrate reduction, and (C) nitrite production/consumption in a mixed methanogenic culture (Initial nitrate concentration equal to 150 mg N/L). Colored figure.

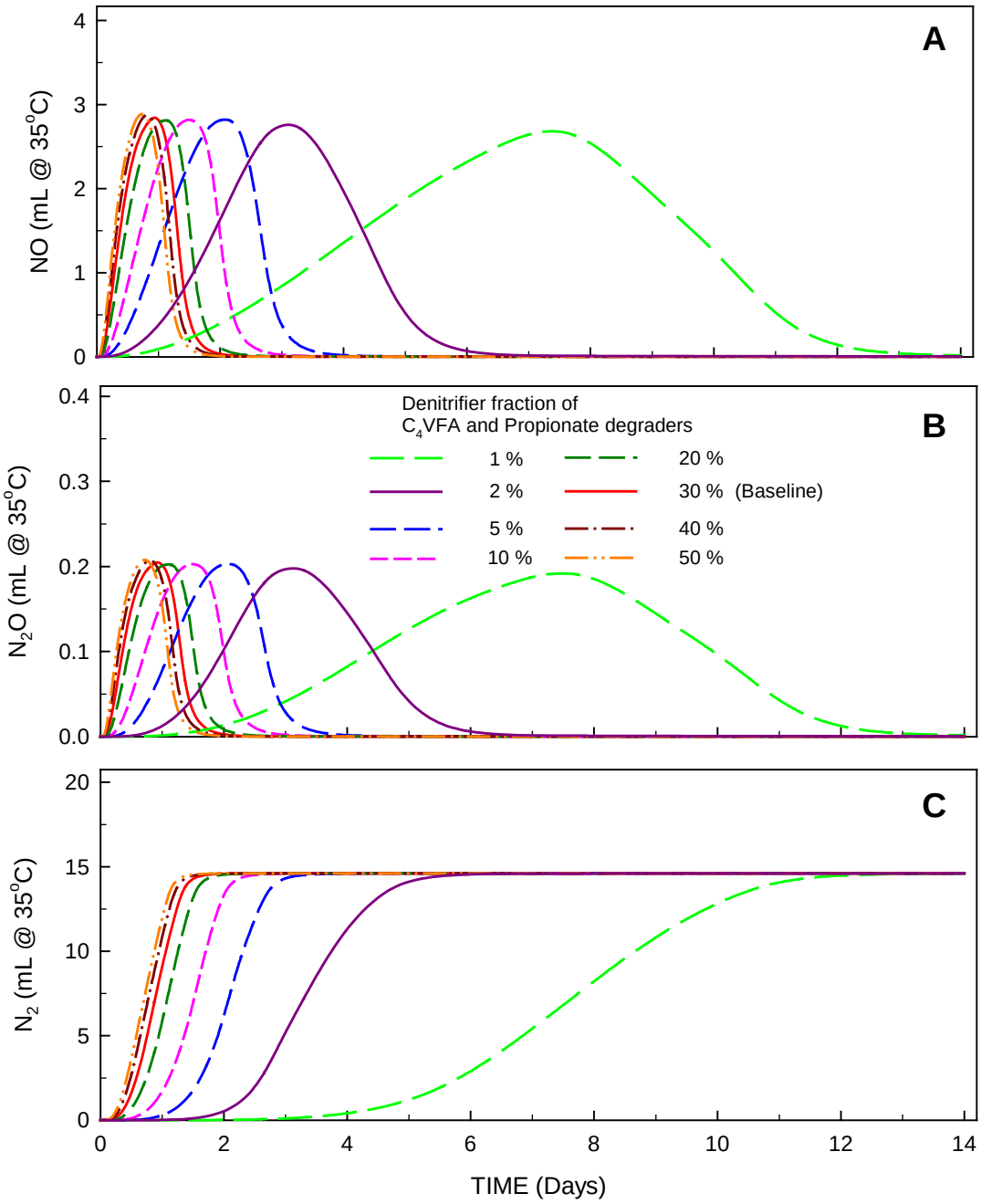

Figure 7.11. Effect of the initial denitrifying population size on production and consumption profiles of (A) NO, (B) N_2O, and production of (C) N_2 in a mixed methanogenic culture (Initial nitrate concentration equal to 150 mg N/L). Colored figure.

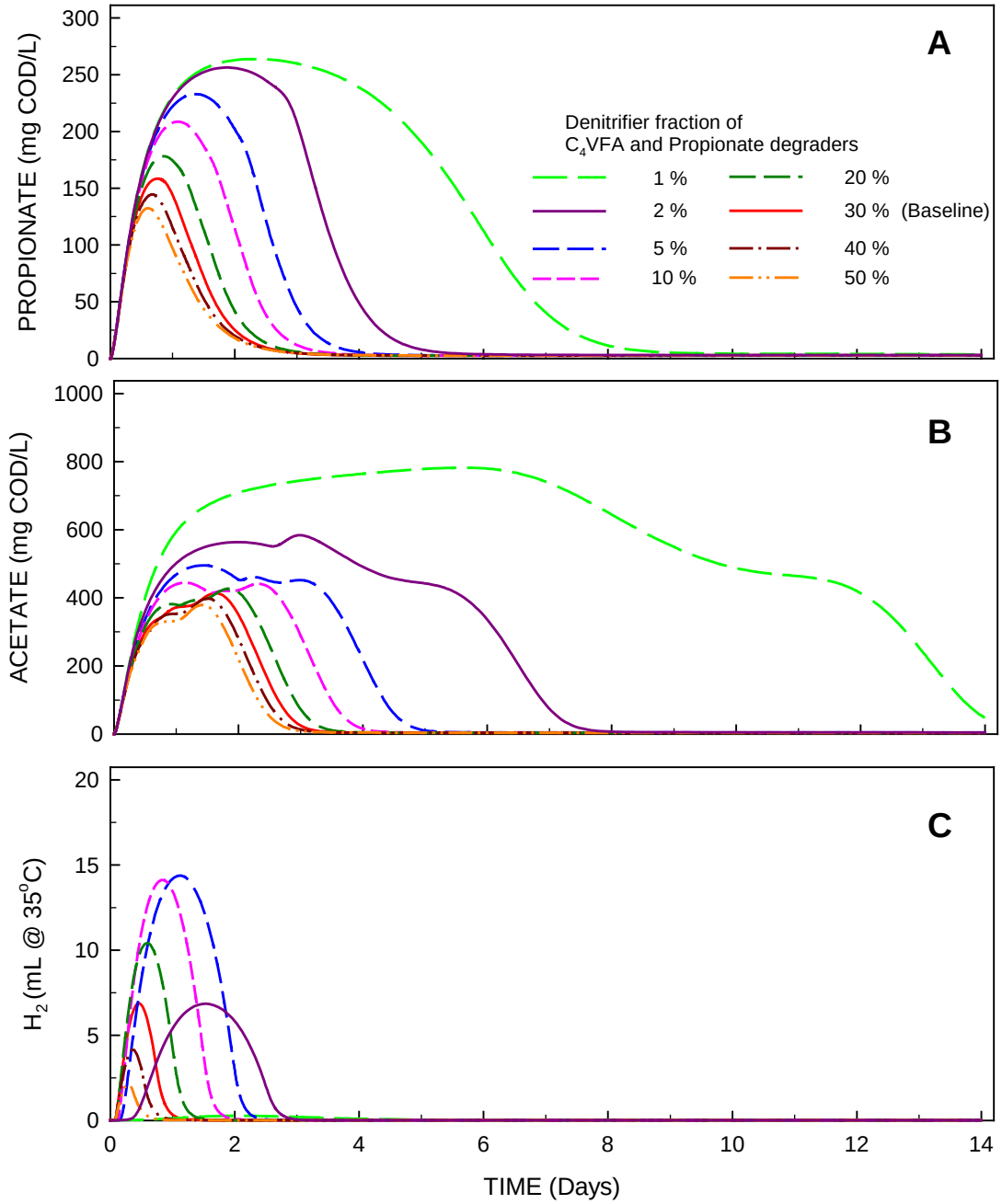

Figure 7.12. Effect of the initial denitrifying population size on production and consumption profiles of (A) propionate, (B) acetate, and (C) H_2 in a mixed methanogenic culture (Initial nitrate concentration equal to 150 mg N/L). Colored figure.

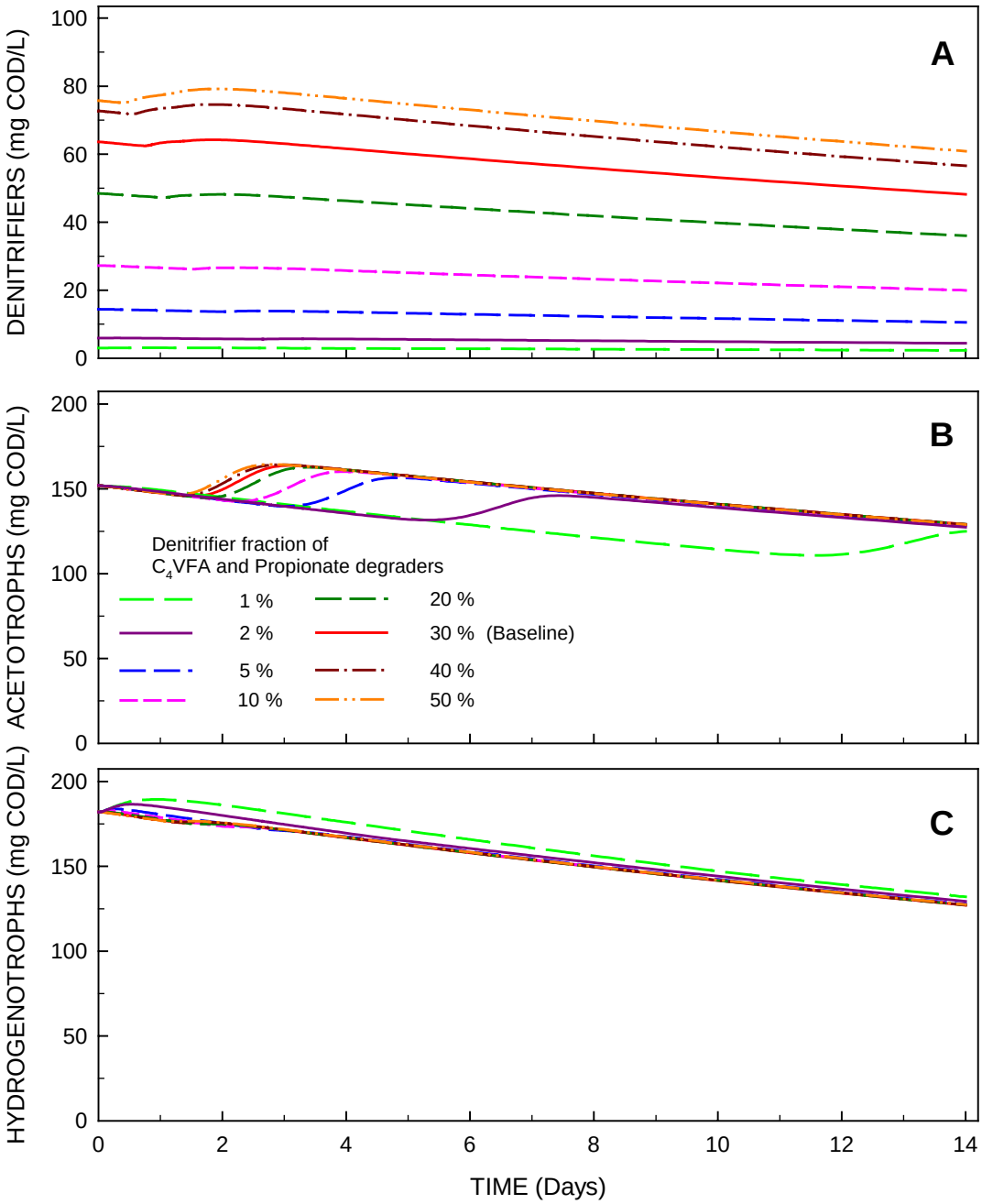

Figure 7.13. Effect of the initial denitrifying population size on growth of (A) denitrifiers, (B) acetotrophs, and (C) hydrogenotrophs in a mixed methanogenic culture (Initial nitrate concentration equal to 150 mg N/L). Colored figure.

7.3.4. Continuous-Flow Simulations

Model simulations were used in order to determine the behavior of continuous-flow systems under different conditions. Specifically, model simulations were used to investigate the effect of nitrate reduction on a mixed methanogenic continuous-flow system at (a) different influent nitrate concentrations, (b) a step increase of influent nitrate concentration from 50 mg N/L to 750 mg N/L, and (c) a step decrease of SRT from 35 d to 15 d. The reactor volume was 1.85 L and the influent dextrin/peptone concentration was maintained at 1000/500 mg COD/L for all cases.

Simulations for all different nitrate concentrations were conducted for an HRT (= SRT) equal to 35 days. In the nitrate-free continuous-flow system, transient accumulation of acetate, propionate, and sugars were observed at the beginning of the simulation run (Figure 7.14). However, the system reached steady-state conditions within 50 days. (Figure 7.14).

When the influent nitrate concentration was equal to 50 mg N/L, the propionate profiles were very similar to that of the nitrate-free system; however, higher acetate levels were observed (Figure 7.15A). In addition, slightly lower methane production was observed. Nitrate and nitrite concentrations were approximately 8 mg N/L and 0.3 mg N/L at steady state, respectively (Figure 7.15B). Low levels of nitric oxide and nitrous oxide productions were observed.

When the influent nitrate concentration was equal to 750 mg N/L, a significantly lower methane production was observed compared to that of the nitrate-free system (Figure 7.16A). In addition, the steady-state effluent nitrate and nitrite

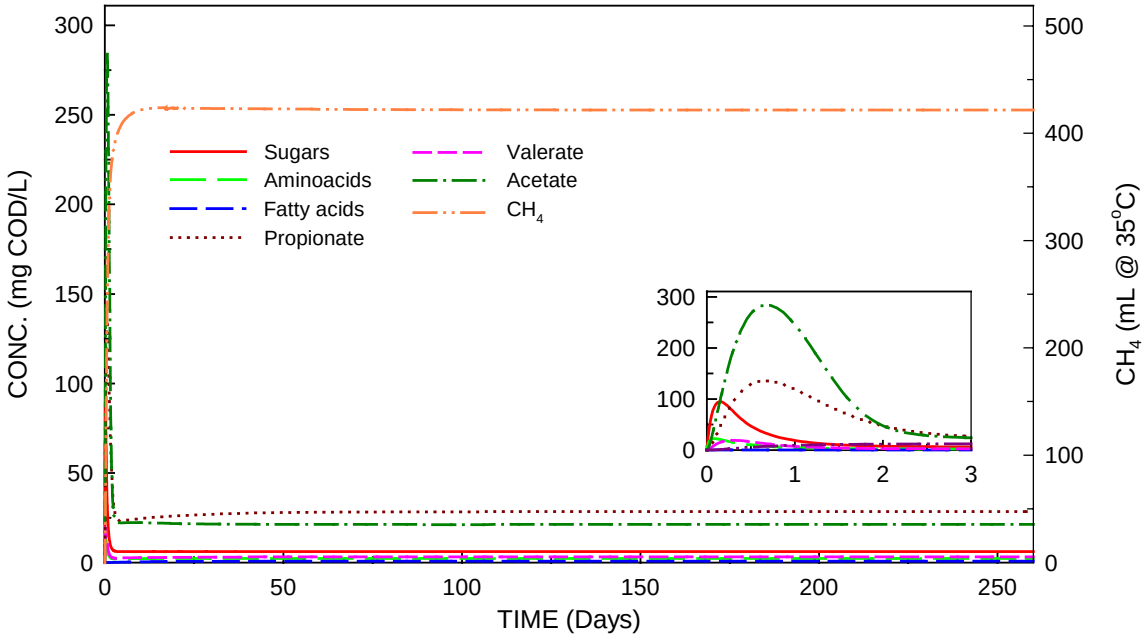

Figure 7.14. Model simulation for a nitrate-free continuous-flow system (V = 1.85 L; HRT = 35 d). Colored figure.

concentrations were 15 and 1 mg N/L, respectively (Figure 7.16B). Higher NO levels were observed in the 750 mg N/L nitrate receiving system compared to that of the 50 mg N/L system (Figure 7.16B).

In order to investigate the effect of a step increase in nitrate concentration, the influent nitrate concentration was increased from 50 to 750 mg N/L at day 200. As a result of the increase in the influent nitrate concentration, a significant increase in acetate concentration was observed (Figure 7.17A). Methane production decreased significantly as a result of the increase in nitrate concentration (Figure 7.17A). In addition, the steady-state nitrate concentration increased to 24 mg N/L as a result of the increase in the influent nitrate concentration (Figure 7.17 B). A slight increase in the nitrite and NO concentrations was observed; however, N_2O levels were not affected.

The effect of a decrease in the HRT from 35 to 15 d on day 200 was investigated. The influent nitrate concentration was 50 mg N/L. As a result of the decrease in HRT, an increase in acetate concentration and methane production was observed (Figure 7.18A). The steady-state nitrate concentration was increased to 15 mg N/L (Figure 7.18B). A slight increase in the steady-state nitrite and NO concentrations were observed.

7.4. Summary

Nitrate reduction processes via denitrification were incorporated into the IWA Anaerobic Digestion Model No. 1 (ADM1) in order to account for the effect of nitrate reduction processes on fermentation and methanogenesis within an overall methanogenic system. The general structure of the ADM1 was not changed, except for modifications related to disintegration and hydrolysis of complex organic matter

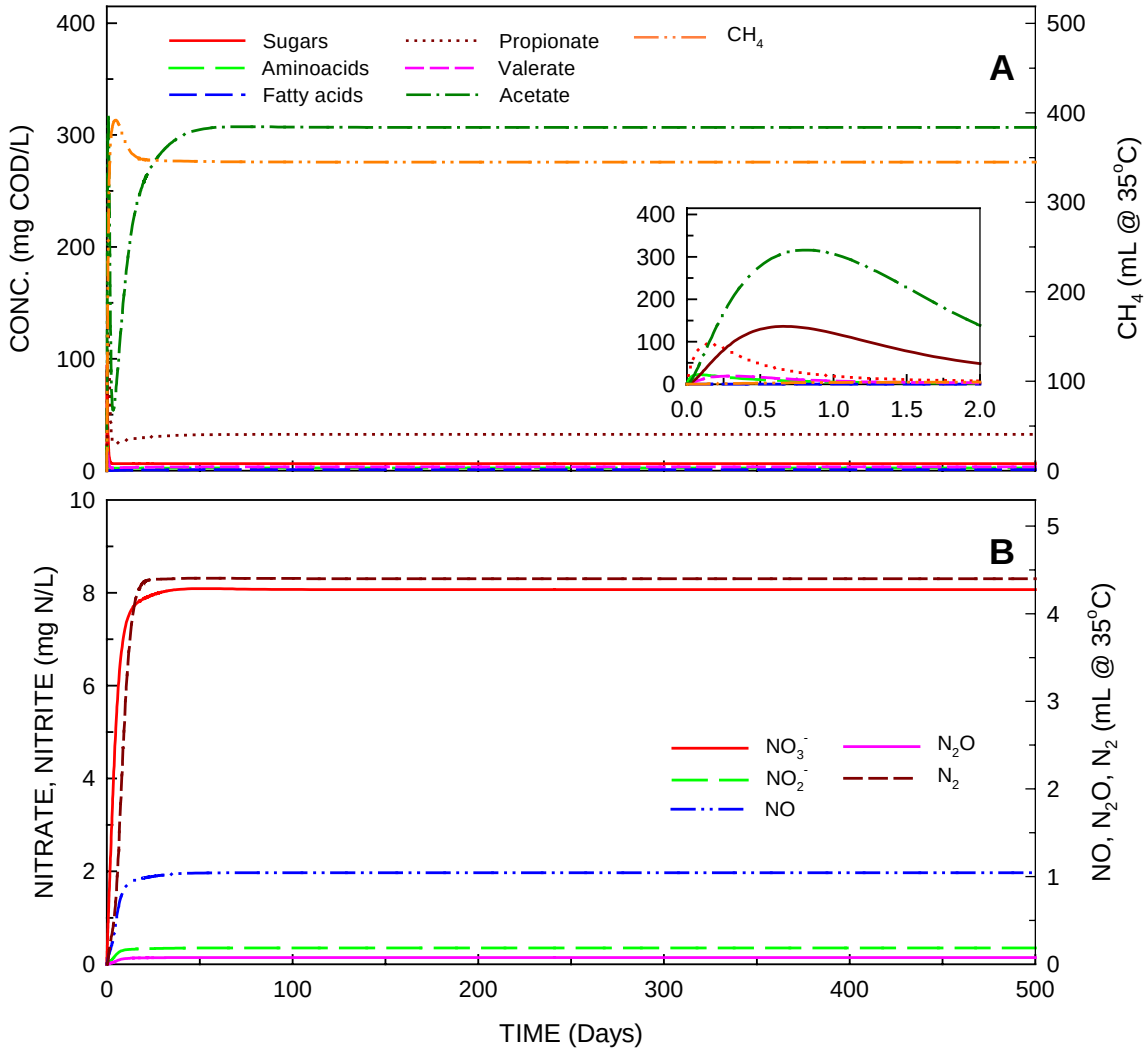

Figure 7.15. Model simulation for a nitrate receiving continuous-flow system (A) Carbon flow and (B) N-oxides (Influent nitrate = 50 mg N/L; V = 1.85 L; HRT = 35 d). Colored figure.

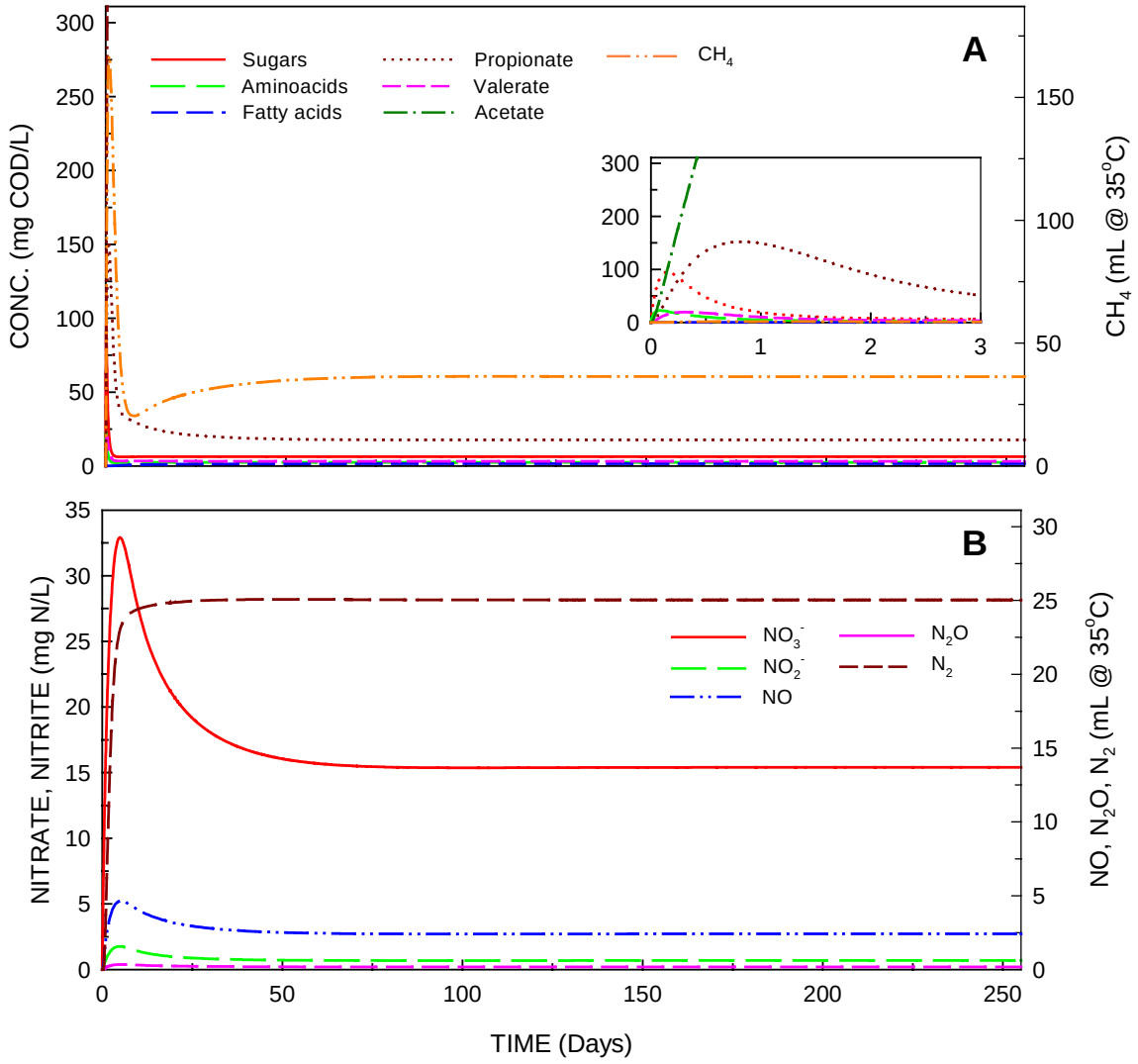

Figure 7.16. Model simulation for a nitrate receiving continuous-flow system (A) Carbon flow and (B) N-oxides (Influent nitrate = 750 mg N/L; V = 1.85 L; HRT = 35 d). Colored figure.

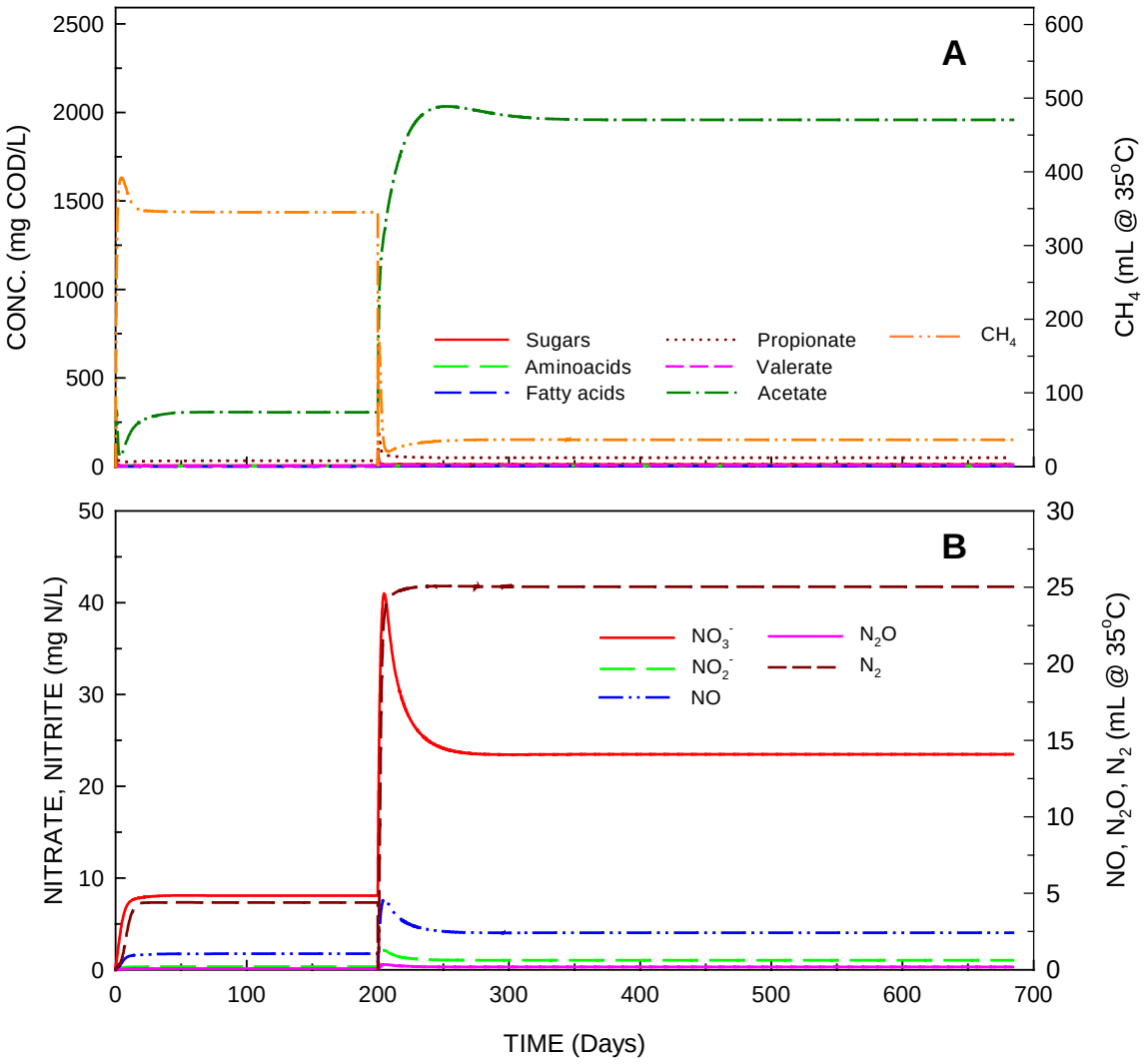

Figure 7.17. Model simulation for a continuous-flow system experiencing a step increase in influent nitrate concentration from 50 to 750 mg N/L.(A) Carbon flow and (B) N-oxides (V = 1.85 L; HRT = 35 d). Colored figure.

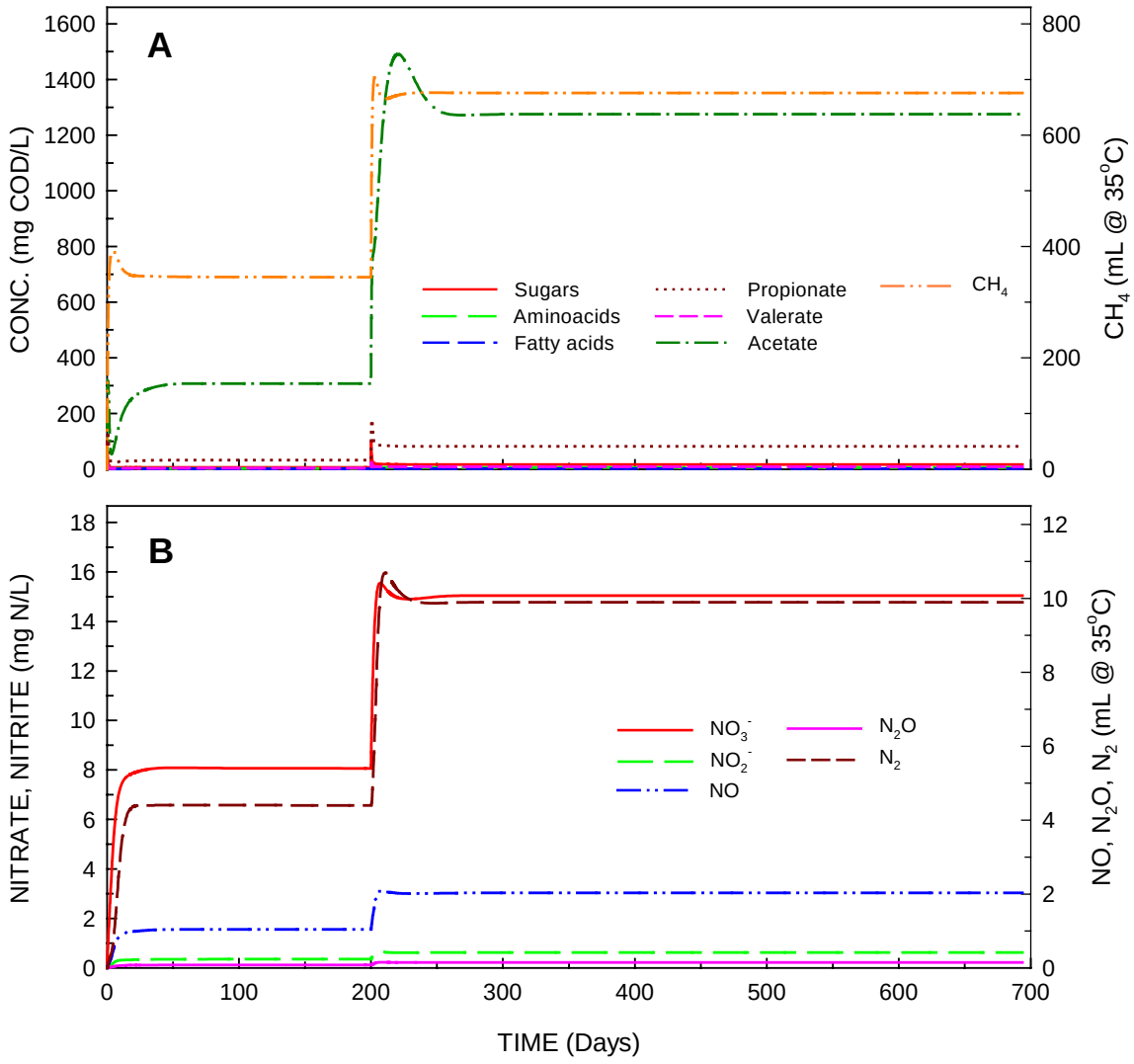

Figure 7.18. Model simulation for a continuous-flow system experiencing a step change in HRT from 35 d to 15 d. (A) Carbon flow and (B) N-oxides (Influent nitrate = 50 mg N/L; V = 1.85 L). Colored figure.

and decayed biomass. A fraction of valerate/butyrate and propionate degraders was assumed to be fermentative denitrifiers carrying out fermentation in the absence of N-oxides. Nitrate reduction proceeded in a stepwise manner to nitrite, nitric oxide, nitrous oxide and nitrogen gas using four substrates as electron and/or carbon source: C_4VFA, propionate, acetate, and hydrogen. The utilization of the four substrates and N-oxides was based on stoichiometry and kinetics. The inhibitory effect of N-oxides on the methanogens was accounted for by the use of non-competitive inhibition functions. Model simulations were compared with experimental data obtained with a batch, mixed fermenting and methanogenic culture amended with various initial nitrate concentrations. Sensitivity analysis revealed that changes in the k_{no3}, K_{no3}, and $k_{no2,pro}$ values had the highest impact on the nitrate reduction rate. Model simulations were used to determine the effect of varying denitrifying biomass fractions on fermentation/methanogenesis and denitrification. Model simulations provided information on the effect of nitrate reduction on methanogenesis in continuous-flow systems when changes in the operational conditions, such as influent nitrate concentration and SRT, occur.

CHAPTER 8

CONCLUSIONS AND RECOMMENDATIONS

The study presented here demonstrated the effect of N-oxides and sulfide, along with the effect of the type of electron donor on the kinetics and pathway of nitrate reduction in a mixed, methanogenic system. The inhibitory effects of nitrogen oxides on a sulfide-free mixed methanogenic culture revealed that the degree of inhibition was a function of the type and concentration of nitrogen oxides, which affected both fermentation/methanogenesis and nitrate reduction. The assays conducted on the effect of sulfide demonstrated that the addition of sulfide affected nitrate reduction differently in sulfide-free enriched and sulfide-acclimated cultures. Abiotic assays showed that microbial activity was necessary for nitrate reduction to occur. Dextrin/peptone, glucose, propionate, acetate, and H_2/CO_2 were used as carbon/electron donor sources and the nitrate reduction kinetics, at different initial nitrate concentrations, were assessed. Process interactions of methanogenesis and nitrate reduction were modeled using a modified and extended ADM1. The ADM1 model was modified in terms of disintegration and hydrolysis processes and extended by the incorporation of nitrate reduction processes.

The following specific conclusions can be drawn based on the results of this study:

1. The inhibitory effect of N-oxides was as follows in decreasing order: NO > N_2O > NO_2^- > NO_3^-. Nitrate addition only affected methanogenesis and did not have any adverse effect on fermentation at the concentrations tested in this study (up to 350 mg N/L). However, nitrite addition caused inhibition of

methanogenesis and fermentation, depending on the initial concentration. Nitric oxide caused inhibition of acidogenesis, fermentation, methanogenesis, and even denitrification at very low initial concentrations (equal or less than 0.8 mg NO/L dissolved).

2. Multiple additions of nitrate to the mixed methanogenic culture resulted in increased rates of nitrate and nitrite reduction, a decreased methane production rate and accumulation of propionic acid in a subsequent, nitrate-free cycle. Long-term exposure of the mixed methanogenic culture to nitrate not only suppressed methanogenesis but also must have changed the relative population composition favoring denitrifiers over fermentative and methanogenic species.
3. Sulfide addition to sulfide-free enriched, methanogenic cultures resulted in inhibition of nitrite, nitric oxide, and nitrous oxide reduction causing accumulation of these intermediates, which in turn inhibited methanogenesis and even fermentation. When mixed methanogenic cultures acclimated to sulfide were amended with nitrate, instead of accumulation of denitrification intermediates, nitrate reduction occurred via DNRA and converted nitrite to ammonia; thus, accumulation of N-oxides was avoided and inhibition of methanogenesis was prevented.
4. The COD/N value plays an important role relative to the fraction of nitrate reduction accomplished via DNRA and denitrification. As the COD/N value increased, DNRA prevailed over denitrification in sulfide-acclimated cultures.
5. The highest rate of nitrate reduction was observed in the H_2- and acetate-fed cultures, whereas, the lowest rates were observed in the propionate-fed cultures. The main pathway of nitrate reduction was denitrification in the H_2-, acetate-, and propionate-fed cultures. Nitrate reduction followed both

denitrification and DNRA in the D/P- and glucose-fed cultures and the predominance of each of the two pathways was a function of the COD/N value.

6. Modification of the ADM1 in terms of disintegration and hydrolysis rates for the feed and decaying biomass enabled the model to better simulate the rate of methane production in a nitrate-free, mixed fermentative/methanogenic culture. The results obtained with the extended model clearly showed the effect of inhibition by intermediate N-oxides on process interactions between fermentation, methanogenesis and denitrification. Model simulations showed that such process interactions cannot be explained based only on stoichiometry and kinetics, especially for batch systems and/or continuous-flow systems with periodic, shock nitrate loads. The model successfully performed for continuous-flow systems at different influent nitrate concentrations and as a function of disturbances in the system operation (i.e., shock nitrate loads, changes in HRT)

The experimental work and model provide a framework in terms of understanding complex anaerobic systems, both natural and engineered. The results obtained in this study can be used to manage and operate anaerobic systems depending on the goal of the process, such as control of methane emissions in natural systems (e.g., flooded rice paddy fields) or methane production in anaerobic digesters. The results obtained in this research are useful in predicting the fate of carbon-, nitrogen-, and sulfur-bearing waste material, as well as in understanding microbial process interactions in both natural and engineered anoxic/anaerobic systems. Because DNRA produces ammonia as opposed to nitrogen gas, the effectiveness of mixed anaerobic systems is reduced in terms of achieving a high degree of nitrogen

removal. Information provided by the present study could lead to the development of strategies to mitigate the negative impact of DNRA, or enhance the production of ammonia, depending on the overall goal of the anaerobic treatment process.

This research revealed the different effects of sulfide on sulfide-free enriched and sulfide-acclimated cultures in terms of inhibition of nitrate reduction and the predominance of either denitrification or DNRA as the main nitrate reduction pathway. However, there is a need for a better understanding of the effect of sulfide at the enzymatic level, to shed light on how the nitrate reduction pathway changes in the presence of sulfide.

The long-tem effect of N-oxides on a mixed methanogenic culture can be further explored by investigating the changes in the microbial population using molecular tools. Understanding the response of the microbial populations to long-term exposure of nitrate may have significant benefits in systems receiving periodical nitrate loads. The kinetics of fermentation/methanogenesis and nitrate reduction can be further explored using mixed, but defined pure cultures, which would allow detailed determination of the process interactions and can be integrated into the extended ADM1. In addition, the DNRA process can be integrated into the extended ADM1 model as a function of sulfide concentration, type of substrate, and COD/N ratio, which would extent the applicability of the model to different systems.

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